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# DETERMINATION OF NICOTINIC ACID IN BIOLOGICAL MATERIALS BY MEANS OF PHOTOELECTRIC COLORIMETRY\*

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(Received for publication, February 12, 1940)

The pyridine nucleus reacts with cyanogen bromide and an aromatic amine to give a yellow compound which can be estimated colorimetrically. This reaction has been made the basis for the chemical determination of nicotinic acid (1-9). In the present study the same reaction has been used for the analysis of biological materials but the errors, due to inadequate extraction or to adsorption, are avoided by direct acid hydrolysis of the test substance, followed by preferential charcoal adsorption for the decolorization of the hydrolysate. For the measurement of the final yellow color resulting from the reaction between nicotinic acid and reagents, a photoelectric colorimeter<sup>1</sup> is used, and in the application of the test to biological materials a new procedure is described for making the blank corrections and for evaluating the effects of interfering substances which modify the sensitivity of the reaction.

Nicotinamide<sup>2</sup> rather than nicotinic acid was used in the re-

\* The expenses of this investigation were defrayed by grants from The Upjohn Company, Kalamazoo, and from the Horace H. Rackham School of Graduate Studies, University of Michigan.

† Upjohn Fellow in Clinical Research, 1937-40.

<sup>1</sup> Obtained from the Rubicon Company, Philadelphia, Pennsylvania. The design and principle of operation of this instrument are described by Evelyn (10). For a more complete description, including the significance of the terms used in the present study, the reader is referred to "Evelyn photoelectric colorimeter, Notes on operation" (1939), Rubicon Company, Philadelphia.

<sup>2</sup> The crystalline nicotinamide was kindly furnished by General Biochemicals, Inc., Cleveland, Ohio. The compound melted at 126°. A



covery experiments. The amide reacts also with the reagents to give the same yellow color but of an intensity corresponding to only one-half of that obtained with an equivalent amount of the acid. Under the conditions of our test the amide is completely hydrolyzed to give the theoretical amount of free acid. This quantitative conversion of amide to acid in tests with the compound added to biological materials is adequate proof that any nicotinamide-containing coenzymes present are also hydrolyzed to yield free nicotinic acid. Von Euler and associates (3) have shown that the union of the pyridine ring to carbohydrate in coenzyme I and II is much more labile to acid hydrolysis than the acid amide group. The present method for the determination of nicotinic acid has been used in the analyses of yeast, liver, rice polish, and wheat germ preparations and of urine, milk, saliva, plasma, and whole blood.

#### EXPERIMENTAL

##### *Reagents for Chemical Reaction—*

Cyanogen bromide. Water saturated with bromine at 5–10° is just decolorized in the cold by the addition of a 10 per cent KCN solution. From 70 to 75 cc. of the KCN solution are used in the titration of 500 cc. of the bromine water.

Absolute ethyl alcohol. This reagent is generally free from pyridine compounds.

Aniline solution. Redistilled aniline is dissolved in absolute ethyl alcohol to make a 4.0 per cent solution.

Standard nicotinic acid<sup>3</sup> solution. This contains 100 micrograms per cc. of absolute ethyl alcohol.

*Results Obtained in Tests with Pure Solutions of Nicotinic Acid—*  
The procedure used in carrying out the reaction is that reported by Shaw and MacDonald (2). To the nicotinic acid in 3 cc. of solution (2 parts of water to 1 part of ethyl alcohol) 6 cc. of the cyanogen bromide reagent are added from a burette. This is

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stock solution was made by dissolving 496 mg. of nicotinamide in 500 cc. of absolute ethyl alcohol. This solution is equivalent on the basis of comparative molecular weights of the solutes to one containing 1 mg. of nicotinic acid per cc.

<sup>3</sup> Obtained from Eastman Kodak Company, Rochester, New York. After recrystallization from hot water, the compound melted at 231°.

followed immediately with the addition of 1 cc. of the aniline solution. The solutions are stirred after the addition of each reagent. The maximal yellow color<sup>4</sup> is then read in the Evelyn photoelectric colorimeter, with Filter 420.

With quantities of nicotinic acid ranging from 3 to 50 micrograms the ratio of photometric density ( $L$  value) to the concentration of the test substance was constant to within  $\pm 1$  per cent. There was a significant positive deviation from the constant  $K$  value when smaller amounts of nicotinic acid were tested, but because of the excellent reproducibility of the results accurate values may still be obtained in the very low range by applying an appropriate correction factor.

*Specificity of Reaction for Nicotinic Acid*—This subject has been more extensively investigated by others (1-6, 9). Their studies indicate that the reaction is specific not for nicotinic acid alone but for the pyridine ring, provided the nitrogen is trivalent. However, the reaction appears to be even more specific than this inasmuch as vitamin B<sub>6</sub>, which also contains the pyridine ring with trivalent nitrogen (11), fails to give a positive color test.<sup>5</sup>

*Reproducibility and Stability of Reagents*—In order to study this problem adequately more than 100 tests with twenty-five different cyanogen bromide reagents and six different aniline solutions were carried out. Representative data are presented in Table I. The  $K$  values obtained with the cyanogen bromide reagents were not constant but varied from 0.215 to 0.259. However, with each of the particular reagents used the  $K$  value was constant in tests conducted with varying quantities of nicotinic acid. This finding indicates that no previously determined reference curve can be used for this determination but that along with each series of tests a  $K$  value should also be obtained. We have preferred in analyses of biological materials to determine for each test solution its own particular  $K$  value. To an aliquot of the test solution 10 micrograms of nicotinic acid are added and the difference in photometric density yields the proper  $K$  value to be used in the calculations. This is necessary since other

<sup>4</sup> This color reaches a maximum in from 3 to 5 minutes and remains constant for at least the next 5 minutes.

<sup>5</sup> The crystalline vitamin B<sub>6</sub> was kindly furnished by Merck and Company, Inc., Rahway, New Jersey.

substances in solution may either inhibit the reaction (*e.g.*, excess of free acid or free alkali) or make it more sensitive (*e.g.*, sodium chloride).

Examination of Table I also reveals that the reagents, when properly stored, are suitable for use for a period of at least 5

TABLE I  
*Reproducibility and Stability of Reagents Used in Chemical  
Determination of Nicotinic Acid*

Reagents		Age of reagents		Storage temperature of CNBr*	Blank center setting, galvanometer reading	Nicotinic acid tested	K value†
CNBr	C <sub>6</sub> H <sub>5</sub> NH <sub>2</sub>	CNBr	C <sub>6</sub> H <sub>5</sub> NH <sub>2</sub>				
		days	days	°C.		γ	
I	I	0	0	5	81 <sup>1</sup>	5	0.262
						10	0.256
						20	0.258
	II	0	75	5	82 <sup>1</sup>	10	0.258
II	III	0	171	5	136 <sup>2†</sup>	10	0.258
	III	0	0	5	80 <sup>2</sup>	5	0.213
						10	0.216
						20	0.215
	III	31	31	5	78 <sup>2</sup>	10	0.218
	II	150	55	5	78 <sup>1</sup>	10	0.206
	III	31	31	25-30	79 <sup>2</sup>	5	0.178
						10	0.179
						20	0.175
	II	150	55	25-30	78 <sup>1</sup>	10	0.072

\* The aniline solutions were stored at room temperature throughout.

† This value corresponds to the photometric density obtained in each test when expressed in terms of 10 micrograms of nicotinic acid.

‡ When the galvanometer in this case was adjusted to 100 with the blank tube, the center setting was not on the galvanometer scale. The recorded value was obtained by adjusting the galvanometer to 50 with the blank tube and multiplying the resulting center setting by 2.

months. This is in contrast to all methods previously published, which advocate the use of freshly prepared reagents. The aniline solutions were stored at room temperature in glass-stoppered, brown glass bottles. The center settings obtained with these solutions and the freshly prepared cyanogen bromide reagent indicate that there is a marked increase in the color of the former

with increasing age. However, this change in the aniline reagent merely alters the center setting without affecting the accuracy of the results. The cyanogen bromide is stored also in a glass-stoppered bottle but in the refrigerator at about 5°. When kept at room temperature the reagent gradually deteriorates with a concomitant decrease in the experimentally determined  $K$  values.

Despite the fact that nicotinic acid is relatively a very stable compound, we have repeatedly observed a rather rapid decrease in its concentration when dilute aqueous solutions (10 micrograms per cc.) are allowed to stand at room temperature. Because the factor responsible for this is bacterial contamination, all standard nicotinic acid solutions are made up in absolute alcohol.

*Use of Preferential Charcoal Adsorption for Decolorization of Nicotinic Acid Solutions*—Analyses of biological materials for nicotinic acid have been complicated by the presence in test solutions of pigments which mask the color produced by the reaction and because of their intensity make impossible colorimetric measurements. Charcoal adsorbs nicotinic acid quantitatively from pure aqueous solutions and to an appreciable extent from biological solutions. For this reason charcoal adsorption has been abandoned and other procedures, though much more tedious, adopted for the preparation of colorless solutions (1-9). However, by making use of preferential charcoal adsorption we have been successful in the decolorization of solutions with no concomitant loss of nicotinic acid.

The validity of this new procedure is indicated by tests with pure solutions of nicotinic acid of known concentration. Variable amounts of charcoal<sup>6</sup> were added to aqueous, strongly acidic, alcoholic, and strongly acidic alcoholic solutions of nicotinic acid. The mixtures were shaken and filtered<sup>7</sup> at room temperature. The filtrates were adjusted<sup>8</sup> in the cold to pH 7 and tests conducted

<sup>6</sup> Darco, a vegetable charcoal, obtained from The Coleman and Bell Company, Inc., Norwood, Ohio.

<sup>7</sup> Whitall Tatum filter paper, manufactured by the Armstrong Cork Company, Lancaster, Pennsylvania, was used.

<sup>8</sup> Concentrated NaOH (18 N) was used to neutralize the acid filtrates with phenolphthalein as an inside indicator. For the final adjustment 1 N NaOH was added with litmus paper as an outside indicator. The phenolphthalein does not influence the reaction and at the pH of the test is in its colorless form.

## 6 Determination of Nicotinic Acid

with 3 cc. samples, containing 2 parts of water to 1 part of alcohol and equivalent to one-tenth of the original solutions. The results, presented in Table II, indicate that quantities of charcoal up to 200 mg. may be added to 25 cc. of the acidic alcoholic solution with no loss of nicotinic acid. Losses from simple acidic or alcoholic solutions are large but the coupling of the two mediums

TABLE II  
*Influence of Solvent upon Adsorption of Nicotinic Acid by Charcoal*

Solvent for nicotinic acid*	Charcoal† added	Nicotinic acid adsorbed
	mg.	per cent
15 cc. H <sub>2</sub> O	100	100
	200	100
	300	100
	500	100
15 " 4 N HCl	100	85
	200	92
	300	92
	500	95
15 " H <sub>2</sub> O + 10 cc. C <sub>2</sub> H <sub>5</sub> OH	100	67
	200	84
	300	88
	500	93
15 " 4 N HCl + 10 cc. C <sub>2</sub> H <sub>5</sub> OH	100	3
	200	0
	300	17
	500	30

\* In each case 100 micrograms of nicotinic acid were added; for the colorimetric tests 10 per cent aliquots were used.

† Darco, a vegetable charcoal, obtained from The Coleman and Bell Company, Norwood, Ohio.

is followed by a synergistic action in the prevention of charcoal adsorption of nicotinic acid.

*Procedure for Evaluating Residual Color in Test Solution and for Converting Photometric Density into Absolute Units of Nicotinic Acid*—For the decolorization of biological solutions the technique of preferential charcoal adsorption is used. No attempt is made to obtain complete decolorization of the alcoholic hydrolysates inasmuch as correction can easily be made for the amount of residual color. The procedure described by Bandier (5) for

making this correction involves the addition of all reagents except the base to an aliquot of the test solution. However, since cyanogen bromide reacts to an appreciable extent with nicotinic acid despite the absence of the base to give the same color, false results are obtained. We have found in tests with pure solutions of nicotinic acid ranging from 3 to 50 micrograms that the yellow color developed without the aniline is equivalent to from 33 to 20 per cent respectively of that obtained when the base is added. It is therefore necessary to carry out an independent blank determination for the residual color subsequent to decolorization in addition to that obtained with the reagents.

The procedure used for making these two independent blank corrections and its validity are presented in Table III. The tests were conducted on 3 cc. aliquots of three 30 cc. nicotinic acid solutions, containing in each case 100 micrograms of the acid in 2 parts of water to 1 part of ethyl alcohol. The solutions were colored to varying degrees by thymol blue indicator. The initial photometric densities were calculated from the galvanometer readings of the samples diluted with a buffer solution<sup>9</sup> to simulate test conditions. The same Filter 420 was used but the center setting was that obtained with a blank tube containing only the pure solvents. Subsequent to the chemical reactions the maximal photometric densities were measured, the center setting obtained with the blank on the reagents being used. By subtracting from the total photometric density that corresponding to the color initially present in the test solution prior to the addition of the reagents, the photometric density resulting from the chemical reaction alone was obtained. For the conversion of this value into micrograms of nicotinic acid use was made of the  $K$  value corresponding to the increase in photometric density when 10 micrograms of nicotinic acid in 0.1 cc. of absolute ethyl alcohol were added to another 3 cc. aliquot of the test solution. The

<sup>9</sup> This solution is composed of 1960 cc. of water, 10 cc. of  $H_3PO_4$  (85 per cent), 30 cc. of 15 per cent NaOH, and 333 cc. of absolute ethyl alcohol. With phenolphthalein as the indicator, 7 cc. of the solution have a titratable acidity equivalent to 10 cc. of 0.05  $N$  NaOH; its pH is 2.4. These values correspond to the titratable acidity and pH of the pooled reagents, 6 cc. of CNBr + 1 cc. of  $C_6H_5NH_2$ , estimated at from 5 to 10 minutes after being mixed at room temperature.

TABLE III

*Determination of Nicotinic Acid in Colored Test Solutions; Experimental Confirmation of Validity of Procedure Used in Making "Double Blank" \* Correction*

Solution No.	Description	Galvanometer reading†		Photometric density‡ of sample	Calculations, nicotinic acid recovered
		Center setting	Of sample		
I	2 cc. H <sub>2</sub> O + 1 cc. C <sub>2</sub> H <sub>5</sub> OH + 7 cc. buffer§	70	100	0.000	$\gamma$
II	Same as I + slight amount of pigment	70	92 <sup>1</sup>	0.035	
III	" " + considerable pigment	70	71 <sup>1</sup>	0.147	
IV	" " + large amount of pigment	70	49 <sup>1</sup>	0.308	
V	2 cc. H <sub>2</sub> O + 1 cc. C <sub>2</sub> H <sub>5</sub> OH + 6 cc. CNBr + 1 cc. C <sub>6</sub> H <sub>5</sub> NH <sub>2</sub>	80 <sup>1</sup>	100	0.000	
VI	10 $\gamma$ nicotinic acid tested in presence of pigment as in II	80 <sup>1</sup>	54 <sup>3</sup>	0.262	$\frac{0.262 - 0.035}{0.229} \times 10 = 9.9$
VII	Same as VI + increment of 10 $\gamma$ nicotinic acid	80 <sup>1</sup>	32 <sup>1</sup>	0.491 ( $K = 0.229$ ) ¶	
VIII	10 $\gamma$ nicotinic acid tested in presence of pigment as in III	80 <sup>1</sup>	42	0.377	$\frac{0.377 - 0.147}{0.229} \times 10 = 10.0$
IX	Same as VIII + increment of 10 $\gamma$ nicotinic acid	80 <sup>1</sup>	24 <sup>3</sup>	0.606 ( $K = 0.229$ ) ¶	
X	10 $\gamma$ nicotinic acid tested in presence of pigment as in IV	80 <sup>1</sup>	29 <sup>1</sup>	0.534	$\frac{0.534 - 0.308}{0.229} \times 10 = 9.9$
XI	Same as X + increment of 10 $\gamma$ nicotinic acid	80 <sup>1</sup>	17 <sup>1</sup>	0.763 ( $K = 0.229$ ) ¶	
XII	0 $\gamma$ nicotinic acid tested in presence of pigment as in IV	80 <sup>1</sup>	49 <sup>1</sup>	0.308 ( $K = 0.229$ ) ¶	$\frac{0.308 - 0.308}{0.229} \times 10 = 0.0$

\* The reason for conducting the two blank tests, one on the reagents and the other on the initial color of the solution, independently is discussed in the text.

† These values are corrected for the slight deviations from true linearity of the relation between current and deflection.

‡ This is analogous to optical density as measured on a spectrophotometer and corresponds to the quantity  $(2 - \log_{10}$  of the galvanometer reading).

§ A 14 per cent alcoholic solution with the same pH and titratable acidity as 6 cc. of the cyanogen bromide reagent + 1 cc. of the aniline solution. In the photoelectric colorimeter it gives the same value as distilled water. Its composition is described in foot-note 9.

|| Thymol blue indicator was used to impart color to the test solutions.

¶ This  $K$  value, calculated by difference, corresponds to the increase in photometric density when the test is carried out with 10 micrograms of nicotinic acid added to an aliquot of the test solution.



validity of these procedures is indicated by the theoretical values obtained with all three nicotinic acid solutions (Table III).

*Determination of Nicotinic Acid in Biological Materials*—The test material, containing from 10 to 400 micrograms of nicotinic acid, is added to a test-tube graduated at the 10 and 15 cc. marks. 5 cc. of concentrated hydrochloric acid (specific gravity of about 1.18) are added and this is followed by distilled water until the total volume measures 15 cc. The acidity is approximately 4 N. The test-tube is immersed in a boiling water bath and the hydrolysis allowed to proceed for a period of from 30 to 40 minutes with occasional stirring. The sample is cooled to room temperature and the volume restored to the original 15 cc. 10 cc. of absolute ethyl alcohol are added and the solution (or suspension) transferred to a 150 cc. Erlenmeyer flask. Exactly 200 mg. of charcoal<sup>6</sup> are added, and the mixture shaken and filtered<sup>7</sup> at room temperature. An aliquot of the filtrate, 8.33 cc., is pipetted into the graduated test-tube, 1 drop of phenolphthalein added, and the solution neutralized<sup>8</sup> in the cold to pH 7. The final volume is brought to the 10 cc. mark.

3 cc. aliquots of the test solution, already containing alcohol in the proper ratio of 1 part to 2 parts of water and equivalent to one-tenth of the original sample, are used for the tests. To the first sample 7 cc. of an alcoholic buffer solution<sup>9</sup> are added. The photometric density of the color remaining after the charcoal decolorization is then estimated according to the procedure described in the preceding section of this paper. A second 3 cc. aliquot of the test solution is used for the chemical reaction and the maximal total photometric density measured. By difference that resulting from the chemical reaction alone is obtained. This value is converted into micrograms of nicotinic acid by use of the  $K$  value obtained when 10 micrograms of the acid are added to a third 3 cc. aliquot of the test solution. The validity of these procedures with examples to illustrate the method of calculation has been presented in Table III.

Representative data obtained in the course of numerous analyses of food sources, rich in the vitamin B complex, and of other materials of biological interest are given in Table IV. The theoretical recoveries of added nicotinamide as nicotinic acid from the hydrolysates support the validity of the procedures used.

In Table IV there is included a column to show what may be considered optimal amounts of the test materials to be used for the analysis. Test solutions, such as saliva and urine, are first concentrated over a steam bath and then brought to volume with the concentrated hydrochloric acid and wash water.<sup>10</sup> In analyses of material, such as milk, plasma, and blood, the concentrated acid is first pipetted into the graduated test-tube. 5 drops of caprylic alcohol are added in order to reduce subsequent foaming. While the acid is being stirred mechanically, the sample is slowly added. The mixture, with the same stirring rod, is then immersed in the boiling water bath. During the first 15 minutes of the hydrolysis the samples are not disturbed. By that time they become sufficiently liquid for adequate stirring.

Alkaline hydrolysis (NaOH) of the test material has been employed by Bandier (4, 5) with good recoveries of added nicotinamide. Such a procedure yields a lighter colored solution than acid hydrolysis. Our own experience with alkaline hydrolysis (2.5 to 9 N), especially of urine, has also shown that added nicotinamide is completely converted to the free acid. However, the yield of "nicotinic acid" from urine samples subjected to such alkaline hydrolysis is far in excess of that obtained by hydrolysis with hydrochloric acid of equivalent normality and increases progressively with increasing concentration of the alkali used. The acid hydrolysis yields constant values despite varying concentrations of the hydrochloric acid used. In all of these cases theoretical recoveries of added nicotinamide are obtained. Furthermore, a normal intake of some materials, devoid of anti-pellagra activity, has been found to augment up to 700 per cent the yield of the substances in urine reacting like nicotinic acid when alkaline hydrolysis (9 N) is employed. Inasmuch as the intake of these substances does not appreciably increase the yield of nicotinic acid obtained by acid hydrolysis, we have preferred to use acid rather than alkali for the hydrolysis of the test mate-

<sup>10</sup> The usual 30 to 40 minute acid hydrolysis is satisfactory for the conversion of all nicotinamide in normal urine to the free acid. Prolonged hydrolysis (5 hours duration) does not increase the yield of nicotinic acid. However, a 5 hour hydrolysis is essential when urine is collected from subjects receiving test doses of extra nicotinic acid. Only under these conditions will the voided nicotinuric acid be completely-hydrolyzed.

TABLE IV  
Determination of Nicotinic Acid in Biological Materials and Validity of Procedures Used

Sam- ple	Description of preparation	Quantity used in analysis	Nico- tin- amide added*	Residual color in aliquot of test solu- tion,† photo- metric density	K value‡ obtained with pure solution	K value§ obtained with test solution	Nicotinic acid found	Recov- ery of added nico- tin- amide*
		mg.	$\gamma$				$\gamma$	per cent
A	Liver extract powder	100	0	0.047	0.244	0.250	115 (1.15 mg. per gm.)	101
B	20% alcoholic liver extract	100	200	0.054	0.244	0.229	317	
		200	0	0.043	0.244	0.236	151 (0.76 mg. per gm.)	108
C	Dehydrated liver and yeast concentrate	200	200	0.037	0.244	0.220	367	
		160	0	0.105	0.240	0.245	194 (1.21 mg. per gm.)	99
D	Dried yeast powder	160	200	0.153	0.240	0.258	392	
		200	0	0.018	0.240	0.260	85 (0.43 mg. per gm.)	101
E	Yeast extract powder	200	200	0.025	0.240	0.243	287	
		100	0	0.014	0.237	0.235	155 (1.55 mg. per gm.)	101
F	Sweetened, concentrated aque- ous extract of yeast	100	200	0.013	0.237	0.217	357	
		200	0	0.076	0.237	0.248	84 (0.42 mg. per gm.)	100
G	Yeast extract powder	200	200	0.047	0.237	0.231	283	
		50	0	0.008	0.229	0.230	126 (2.52 mg. per gm.)	97
H	Aqueous concentrate of rice polish extract	50	200	0.008	0.229	0.223	320	
		25	0	0.011	0.240	0.234	71 (2.84 mg. per gm.)	99
I	Wheat germ powder	25	200	0.013	0.240	0.226	269	
		300	0	0.067	0.233	0.258	45 (0.15 mg. per gm.)	102
		300	200	0.090	0.233	0.245	248	

J	Human saliva	cc.	0	0.014	0.238	0.241	17 (0.07 mg. %)	85
K	" urine (24 hr. sample = 1340 cc.)	30	100	0.018	0.238	0.240	102	
L	Cow's milk¶	28 (4 hr.)	0	0.153	0.233	0.238	77 (3.7 mg. per 24 hrs.)	99
		28 (4 " )	100	0.132	0.233	0.244	176	
		10	0	0.176	0.244	0.277	45 (0.45 mg. %)	100
M	Human plasma¶	10	200	0.177	0.244	0.256	245	
		10	0	0.126	0.254	0.274	13 (0.13 mg. %)	103
		10	100	0.115	0.254	0.272	116	
N	" oxalated blood**	10	0	0.182	0.218	0.247	72 (0.72 mg. %)	98
		10	100	0.206	0.248	0.233	170	

\* Expressed in terms of nicotinic acid. The amido was added as such but recovered as the free acid.

† This represents the amount of pigment remaining in a one-tenth aliquot of the test solution subsequent to charcoal decolorization. Such an aliquot is used for the chemical test. The term photometric density is analogous to optical density as measured on a spectrophotometer and corresponds to the quantity  $(2 - \log_{10}$  of the galvanometer reading).

‡ This value corresponds to the photometric density resulting from the chemical reaction between 10 micrograms of nicotinic acid in pure solution and the reagents.

§ This K value, calculated by difference, corresponds to the increase in photometric density when the test is carried out with 10 micrograms of nicotinic acid added to another one-tenth aliquot of the test solution.

¶ Consistent low recoveries of 85 per cent of added nicotinamide are obtained in tests with saliva. These low values are not due to any destruction of nicotinamide by saliva or to adsorption of nicotinic acid during charcoal decolorization. Because the loss is not an absolute one but a constant fraction of the nicotinamide added, the found value of mg. per cent of nicotinic acid in saliva has been corrected for this lost fraction.

¶ One 200 mg. charcoal addition is employed for the decolorization of each of the test solutions, Samples A to K. In Samples L and M an additional treatment with 100 mg. of charcoal is carried out to obtain satisfactory decolorization.

\*\* For the decolorization of the alcoholic hydrolysate of whole blood three charcoal additions of 300 mg. are employed. 40 mg. of potassium oxalate powder are used as the anticoagulant for 10 cc. of blood.

rials. A more detailed and extended report of these findings will be the subject of a subsequent paper.

In the fifth column of Table IV, the amount of color remaining in an aliquot of each of the test samples subsequent to charcoal decolorization and neutralization is indicated. The specimens from Samples A to K contained very little color. In the case of Samples L to N the decolorization was not satisfactory when the usual single addition of 200 mg. of charcoal was used. Additional charcoal treatments were essential. In the analysis of materials such as milk and plasma the initial charcoal adsorbate is removed by centrifugation and an additional 100 mg. quantity of charcoal added to the decanted solution. The mixture is then filtered. With whole blood three additions of 300 mg. of charcoal are employed to decolorize the alcoholic hydrolysates. Because substances in biological solutions inhibit adsorption of nicotinic acid, amounts of charcoal can be used for the decolorization of such solutions in excess of the maximum found in tests with pure acidic alcoholic solutions of nicotinic acid (see Table II). The excellent recoveries of the nicotinamide added to Samples L to N support the validity of this new procedure of stepwise preferential charcoal adsorption for the decolorization of solutions with no loss of nicotinic acid.

In Table IV comparison is made between the  $K$  values obtained in tests with 10 micrograms of nicotinic acid in pure solutions and those calculated by difference when the 10 microgram quantities of nicotinic acid were added to aliquots of the test solutions. The discrepancies between these two series of  $K$  values are real and do not represent errors in the analyses. The variations in these values among the different test solutions are due to the presence of other substances in varying concentration in these solutions which tend to modify the sensitivity of the reaction. However, in any given case the  $K$  value is constant over the range of nicotinic acid concentration determined by the present method. This was indicated by additional tests with graded amounts of nicotinic acid (from 1 to 50 micrograms) added to the *same* test solution.

Von Euler and associates (3) have pointed out that the color produced by the reaction of the reagents with nicotinamide is fully 4 times as great as that obtained with the dihydropyridine remaining after acid hydrolysis of the reduced codehydrogenases.

They state that these compounds are completely oxidized by atmospheric oxygen during simple aqueous extraction and that solutions of the reduced codehydrogenases are obtained only by alkaline extraction. The yield of nicotinic acid from the test materials of this study was not increased by preliminary aeration<sup>11</sup> in the presence of hydrogen peroxide at pH 7 and at 37° for 30 minutes.

#### SUMMARY

Nicotinic acid reacts with cyanogen bromide to give a yellow color, the intensity of which is increased 3- to 5-fold by the addition of aniline. The ratio of maximal photometric density, measured by the Evelyn photoelectric colorimeter, to the quantity of the acid (from 3 to 50 micrograms) is a constant. With application of the proper correction factor as little as 1 microgram of nicotinic acid may be determined. The reagents, when properly stored, are suitable for use for a period of at least 5 months. The chemical reaction has been used in the analyses of yeast, liver, rice polish, and wheat germ preparations and of urine, milk, saliva, plasma, and whole blood. The test material is subjected to direct acid hydrolysis, followed by preferential charcoal adsorption for the decolorization of the solution with no concomitant loss of nicotinic acid. A new procedure is described for the estimation of the photometric density due solely to the reaction between nicotinic acid and reagents and for the conversion of this value into absolute units of the acid. The necessity for using acid in preference to alkaline hydrolysis is indicated. Oxidation prior to hydrolysis does not appear to be essential.

*Addendum*—While this paper was in press, the report by Harris and Raymond (13) appeared, advocating the addition of aniline to the blank test. The base was found to react directly with substances in the hydrolysates to give colors indistinguishable from that obtained in tests for nicotinic acid. We had also found this to be true. However, our more extensive studies indicated conclusively that in the presence of cyanogen bromide

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<sup>11</sup> In the case of the whole blood samples complete hemolysis was effected in one series by simple dilution with water, in the other by the addition of saponin. The reason for this preliminary treatment is the fact that the nicotinamide-containing coenzymes of the blood are entirely in the cells (12).

these interfering side reactions do not occur. The details of these studies are now in press. Accordingly, we omitted aniline from the blank test and used the procedure described in this paper for evaluating residual color in the hydrolysate.

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# THE IRON CONTENT OF THE TISSUES OF NORMAL, ANEMIC, AND IRON-ENRICHED RATS FREED FROM BLOOD BY VIVIPERFUSION

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Data on the iron content of tissues and organs in varying metabolic states are scanty. Whipple and his associates (1, 2) have reported the analysis of the tissues of viviperfused dogs. No similar data are available on the rat. Wakeham and Halenz (3) have published values of the iron contents of tissues of normal and anemic rats, washed to free them of blood. This procedure is probably not so effective as viviperfusion in freeing the tissues of blood. Because of the high iron content of blood, Whipple and coworkers have emphasized the importance of freeing the tissues of their contained blood by viviperfusion in order to obtain reliable estimates of their cellular iron content.

To perfuse the rat is quite difficult because of the small blood vessels of this mammal. In spite of this difficulty, a successful operation has been developed by the authors for the viviperfusion of the rat by introduction of a cannula in the external jugular vein. With proper care, the operation yields an adequate perfusion.

The viviperfusion technique was developed in order to pursue a study of the metabolism of iron, with the radioactive isotope  $\text{Fe}^{59}$  as a tracer. Because of the paucity of published data, a series of analyses was carried out on the iron content of the tissues of normal, anemic, and iron-enriched rats freed from blood by viviperfusion.



*Viviperfusion Technique*

The aim of the operation is to free the organs and tissues of blood by perfusing the vascular system with a modified Locke's solution, respiration and circulation being maintained until the blood is completely displaced. The modified Locke's solution was

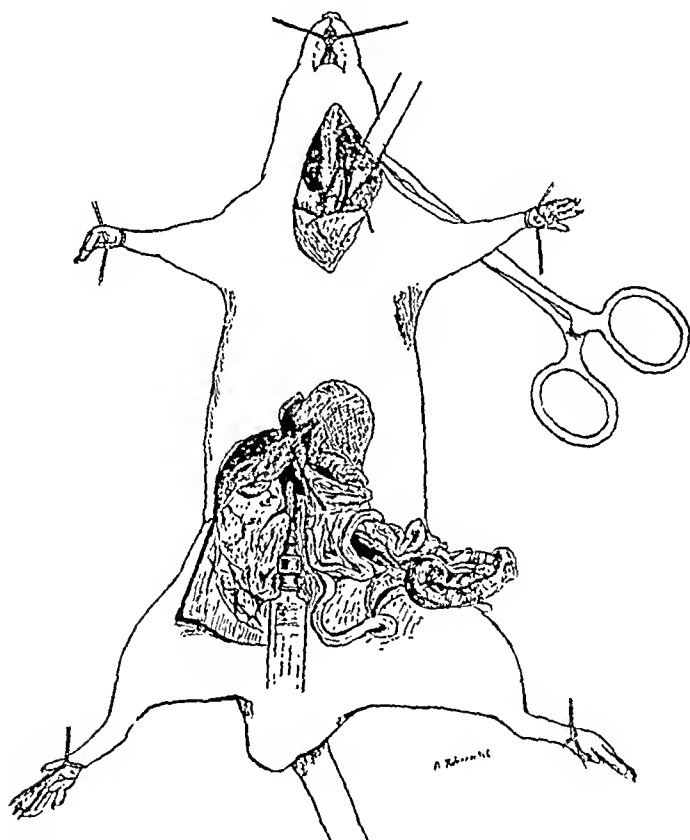


FIG. 1. Diagram of rat showing injection of heparin and insertion of cannula for viviperfusion.

composed of NaCl 0.92 per cent, KCl 0.042,  $\text{CaCl}_2$  0.024,  $\text{NaHCO}_3$  0.015, and glucose 0.1.

To carry out the operation, the rat is anesthetized with nembutal (dosage 4 mg. per 100 gm. of body weight by intraperitoneal injection) and is tied down on the operating board, as shown in Fig. 1. The skin and connective tissue are cut on a mid-line over the larynx, and the salivary glands are separated from each other.

Then either the left or right gland is pulled aside by gently severing the connective tissue fibers, care being taken not to sever any important vessels. The tip of the gland and the skin are pinched together with a hemostat, and the latter is laid down so as to maintain a wide opening. The external jugular vein and its two upper branches are isolated for as great a length as is possible. This is done without cutting the muscles, as they help to hold the cannula in place.

At this point the abdomen is slit longitudinally from the pubis to the thorax without touching the diaphragm, and a transverse cut is made toward the right side of the animal. The abdominal cavity is now cleared by delicately lifting out the viscera and placing them on the animal's left side. This exposes the inferior vena cava, which is a comparatively large vessel. The anti-coagulant, heparin, is injected into the vena cava through a hypodermic needle. The needle is left in the vessel, the viscera are put back in place, and the opening is temporarily closed with a hemostat to prevent drying.

The blood having been made incoagulable, the cannula is now inserted. A strong silk thread is passed under the jugular just above the clavicle, and the lumen of the vessel is collapsed by clamping the ends of the thread with a hemostat. Thin bulldog clamps are used to pinch off the two upper branches of the jugular. The vessel is grasped with a small forceps, and the jugular is slit at the point held by the forceps, as close as is possible to the branching of the jugular. The blood is wiped away and, with the right hand, a cannula is introduced as far toward the heart as possible, while the vessel is held by the forceps in the left hand. The hemostat holding the thread is then released, and the cannula temporarily tied in place with a single knot. The cock controlling the perfusion fluid is turned to allow a little of it to flow into the vein, as a test that the cannula has been inserted into the lumen. If there is no flow, one may try to direct the cannula so that its axis is coincident with the direction of the jugular as it dips toward the heart. When a good flow of saline is established, the cannula is fastened in place with a second knot.

The hemostat closing the abdominal opening is now removed and the secondary and tertiary veins (iliac, femoral, etc.) are punctured. The hind limbs are released so as to allow a good circulation.

After 5 to 15 minutes perfusion, the heart may stop. It can be started again by opening the thorax along the mid-line and injecting a few tenths of a ml. of 1:1000 adrenalin solution into the heart cavity, and gently massaging the heart muscle to stimulate the ventricular contraction.

The Straub single tube cannula, shown introduced into the jugular in Fig. 1, has the disadvantage of allowing a little air from the tip to flow into the circulation, producing small embolic hemorrhages here and there in the lungs.

The cannula is filled with the Locke's solution before being attached by rubber tubing to the saline reservoir. Air can be detached and withdrawn with a needle and syringe, by introducing the needle through the rubber tubing. The Locke solution reservoir consisted of a Woulfe bottle, with an outlet at the bottom. It was inverted and fixed in place in an inverted Bell jar with a tubulated top, through which the tube feeding the Locke's solution could be passed. Warm water was circulated through the Bell jar to maintain the Locke's solution at body temperature.

For the present work a group of animals was perfused on the same day, and the best organs were selected for chemical analysis. Imperfectly perfused tissues were discarded. Sometimes in an animal otherwise well perfused, a single organ, such as the lungs or testes, would be imperfectly perfused. In the lungs this may be due to gaseous embolisms, and in other tissues to regions of peripheral vasoconstriction that retard the flow of saline.

Perfusion of the liver and other organs in the abdominal viscera can be greatly improved by injecting Locke's solution under considerable pressure into the thoracic aorta and massaging the spots where blood still appears to be present.

The general degree of perfusion of the different tissues and organs, their appearance, and the specific treatment that was followed in each case are briefly described below.

*Lungs*—If introduction of air into the vessels while the cannula is held in place is avoided, and the circulation has been maintained for a sufficient time, the lungs appear perfectly white. Pink spots occasionally may be present owing to hemorrhages resulting from gaseous embolism.

*Heart*—In a well perfused heart, the muscle appears decolorized and the coronary vessels are full of Locke's solution.

*Liver*—Adequate perfusion turns the liver a whitish brown color. The tissue becomes quite soft through imbibition of saline. Perfusion of the liver can be greatly improved by injecting Locke's solution into the portal vein and massaging the spots where blood still appears to be present.

*Kidney*—This organ turns a whitish brown color and a perfectly clear saline solution flows from the open renal veins.

*Testes*—These are quite difficult to free from blood. However, if they are brought out from the scrotum, they may be freed from a considerable quantity of blood by injections of saline into the abdominal aorta. On good perfusion, the testes will look almost completely white and, on removal of the capsule, the parenchyma will appear faintly pink. Occasionally, the main blood vessels still contain diluted blood, but these may be dissected out.

*Brain*—This organ appears white, and the meningeal vessels are free of blood.

*Bone*—The evaluation of the extent of perfusion in the bones is quite difficult; however, it can be presumed that a moderate degree of perfusion is attained. The diaphyses of the tibia and femur were taken apart after perfusion, and freed of bone marrow by means of a large syringe attached to the bone with transparent rubber tubing. The bone marrow is then washed away with an acidulated saline solution. The bones are sectioned with bright chrome-plated scissors, and freed, as much as possible, from bone marrow and periosteum, and finally washed again with saline.

*Bone Marrow*—Perfused bone marrow appears lighter than the non-perfused. However, the degree of perfusion is not complete. For analysis the bone marrow is collected in very small crucibles.

*Spleen*—After a thorough perfusion and injection of saline into the thoracic aorta, this organ appears paler than usual, and the vessels approaching it are free of blood.

*Intestinal Tract*—A good perfusion completely frees all of the intestinal tract and the mesentery of blood. The injection of saline into the thoracic aorta improves the quality of the perfusion. Sections not completely free of blood may be separated from the rest of the small intestine. For analysis, the intestinal tract is separated from the mesentery, slit open, and the contents thoroughly washed out.

*Muscle*—After a good perfusion and injection of saline in the

lower aorta, the muscles of the thoracic walls appear whitish pink. The deeper muscles are less well perfused and are a deeper color. They were not used in the analysis.

#### EXPERIMENTAL

The iron was determined by oxidimetric titration with titanium trichloride, according to the method of Klumpp (4). Following perfusion, the tissues and organs intended for analysis were dissected out with bright chrome-plated instruments, and further washed by being placed in silica ware crucibles and covered with saline solution. Small cuts were made into the organs to allow for better washing. After about an hour, the saline was removed, the tissues rinsed, and the crucibles and contents placed overnight in an electric oven kept at between 90–100° in order to dry. After drying, the crucibles and contents were weighed, and the tissues were dry-ashed in an electric muffle. Jackson (5) has pointed out that there is a possibility of loss of iron in the form of ferric chloride by dry ashing if the temperature is elevated too high. In the present work the initial temperature was maintained at 360° and was then allowed to rise slowly up to 700°. After from 12 to 20 hours ashing, the samples were allowed to cool, and the ash was then dissolved with small portions of 6 N HCl, heat being employed to complete the solution of the ash in the acid.

Difficulties due to the presence of copper were encountered only with the liver, and only in this organ was the copper removed by precipitation with  $H_2S$ , according to the procedure of Klumpp (4). A blank titration was run on all reagents to test for the possibility of iron contamination.

The animals used for analysis consisted of normal rats from our stock colony, rats made anemic by being reared on a modified skim milk diet,<sup>1</sup> and iron-enriched rats. Iron enrichment was attained by feeding 1 mg. of Fe as ferric chloride daily to each rat, and keeping them at the reduced atmospheric pressure of 300 to 400 mm. of Hg for about 1 month in the low pressure tanks of Professor E. S. Sundstroem (6).

<sup>1</sup> 84 per cent powdered skim milk (Klim), 15 per cent Crisco, and 1 per cent cod liver oil. 1 mg. of crystalline thiamine chloride was added per 100 gm. of diet. The Fe content was from 4 to 7 mg. per kilo of food.

*Analytical Results*

Viviperfusion generally causes some increase in the water content of the tissues. This makes analytical values based on wet weight somewhat unreliable. Whipple and coworkers (1, 2) recognized this fact in their work, but regarded the increase in

TABLE I

*Influence of Perfusion on Fresh Weights of Tissues from Three Male Rats, 1 Year Old, Weighing 500 Gm. Each*

Organs	Water content		
	Found	Reported by Donaldson (7)	Difference
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Bone marrow	63.8		
"	40.0	33.6	+6.4
Heart	82.3	77.6	+4.7
Stomach	78.4		
	81.7		
Small intestine	86.8		
	83.1		
Large "	84.2		
	80.1		
Kidney	83.4	77.1	+5.3
	82.0	77.1	+4.9
Spleen	76.9	77.4	-0.5
Liver	73.3	74.0	-0.2
	75.3	74.0	+1.3
Lungs	80.3	81.6	-1.3
	80.0	81.6	-1.6
Testes	87.2	87.0	+0.2
Muscle	79.5	76.2	+3.3
	76.8	76.2	+0.6
Brain	80.6	77.5	+3.1
	79.6	77.5	+2.1

water content as a negligible factor with respect to the accuracy of the analysis. A comparison of the percentage water content of the tissues of three perfused male rats with data for the water content of similar animals, compiled by Donaldson (7), is given in Table I. The results in Table I show a water increase in most of the tissues on which comparison could be made. It is interesting

to note that the liver, which appeared to become water-logged and mushy during the perfusion, showed little water gain.

The analytical values for the iron content of the tissues of normal, anemic, and iron-enriched rats are summarized in Table II. The figures are reported both on a dry weight and moist weight basis. The dry weight values were obtained directly from weighing the dry tissues, and the moist weight values were calculated from the dry weights, the water content given by Donaldson being used when it was available and the water content obtained in the series shown in Table I for tissues on which data in Donaldson's work were not available.

The trend of the values in Table II indicates that rats on diets deficient in iron show decreased iron in the tissues as compared to normal rats on the stock diet, while rats on diets supplemented with iron, and kept under reduced pressure, showed, in general, increased iron in the tissues.

The data are in good agreement with published values of Whipple and coworkers (1, 2) for the iron content of similar tissues in the dog. They are of the same order of magnitude, but generally somewhat lower than those reported by Wakeham and Halez (3). This might be expected, since the method of washing used by the latter authors is probably not so efficient as viviperfusion for freeing the tissues of blood.

Because of the variability found in the iron content of tissues (Table II) and the small series analyzed, only large differences in the mean values between normal, anemic, and iron-enriched rats are probably significant. The great variation in the iron content of tissues has been emphasized by Hahn (8).

Most strikingly depleted of iron by the anemia are the liver and spleen. Significant, but lesser, degrees of depletion are shown by the bones, heart, brain, and lungs. Much evidence is available that the liver and spleen have important functions in iron metabolism (8, 9). It is interesting that the analysis of the bone marrow, also an important factor in iron metabolism, shows no significant alteration in the iron content of the anemic animals. A possible explanation for the maintenance of the iron content of the anemic bone marrow is that there is a mobilization of the iron of the body into the bone marrow for forced hemoglobin formation.

Attempted enrichment of the body iron by iron feeding led to

TABLE II  
*Iron Content (in Mg. per 100 Gm.) of Tissues of Normal, Anemic, and Iron-Fed Rats*

Tissue	Normal (7 rats, 200-300 gm. weight, 14.4-15.2 gm. Hb per 100 ml. blood)			Anemic (9 rats, 140-225 gm. weight, 7.0-12.7 gm. Hb per 100 ml. blood)			Iron-fed (5 rats, 200-300 gm. weight, 14.4-15.2 gm. Hb per 100 ml. blood)		
	Dry		Wet	Dry		Wet	Dry		Wet
	Range	Mean		Range	Mean		Range	Mean	
Bone marrow	47.3-58.5	54.4	17.2-21.1	34.2-75.8	55.2	12.4-27.0	87.5-155.3	125.5	31.0-56.4
Liver	11.1-33.6	21.3	2.0-8.7	8.0-10.6	9.3	2.1-2.7	17.7-24.1	20.1	4.2-6.2
Spleen	97.7-237.0	177.5	20.1-64.7	24.2-105.2	73.2	5.4-21.3	94.5-270.5	180.4	19.4-61.1
Kidney	17.9-20.9	19.1	2.1-6.1	11.3-20.6	17.0	2.5-4.3	17.3-37.6	30.1	3.9-8.2
Muscle	5.8-10.1	7.9	1.3-2.4	4.7-10.7	6.9	1.1-2.6	7.0-11.6	9.8	1.0-2.5
Heart	24.4-48.6	33.2	5.0-11.1	19.3-36.7	27.1	4.2-7.9	34.0-52.7	44.5	7.5-11.1
Brain	13.3-16.0	14.9	3.3-4.2	5.4-19.1	10.5	1.1-4.2	19.1-23.8	21.0	4.3-5.1
Testes	12.0-16.1	14.9	1.6-2.0	5.0-23.0	12.2	0.9-5.9	23.2-45.0	30.9	2.8-5.9
Lungs	12.0-31.5	21.9	2.1-5.8	9.0-17.9	14.8	1.7-3.3	28.7-54.9	39.0	5.2-10.4
Bones	1.5-6.3	4.2	1.0-4.1	1.4-3.2	2.0	0.9-2.0	2.5-8.7	4.6	1.0-5.4
Stomach	8.8-12.5	10.4	1.8-2.8	6.2-12.7	8.8	1.5-2.7	9.0-20.8	16.4	2.4-5.3
Small intestine	5.2-25.8	12.2	0.5-3.4	11.4-20.8	15.3	1.9-3.3	7.4-23.2	14.0	1.2-10.7
Large "	6.0-20.0	13.5	1.3-3.1	4.1-11.3	6.9	0.8-2.2	9.6-15.2	13.3	1.9-7.6



significant increases in the iron content of bone marrow. The tissues that were most easily depleted, namely the liver and spleen, registered no gain.

#### SUMMARY

1. An operation has been devised for viviperfusion of the rat by which it is possible to obtain practically blood-free tissues for the estimation of the iron content of the tissue.

2. Analyses have been carried out on the iron content of the tissues of normal, iron-deficient, and iron-enriched rats.

3. The data, in general, show a decrease in the iron content of the tissues of anemic rats, the liver and spleen being most strongly depleted.

4. Iron enrichment leads to an increase in the iron content of most of the tissues, the bone marrow showing the greatest gain. Iron enrichment did not significantly increase the iron content of the liver and spleen.

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# STUDIES IN IRON METABOLISM WITH THE AID OF ITS ARTIFICIAL RADIOACTIVE ISOTOPE

## THE ABSORPTION, EXCRETION, AND DISTRIBUTION OF IRON IN THE RAT ON NORMAL AND IRON-DEFICIENT DIETS

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The discovery of artificially induced radioactive isotopes (1) and their easy preparation through the development of the cyclotron by Lawrence and collaborators (2) have provided a powerful tool for the study of metabolism through the use of "labeled" molecules. This tool has been successfully applied to the study of many of the biologically important mineral elements (3). The recent production of radioactive iron (4) offers the promise of employing this new technique as an aid in clearing up the enormous, but contradictory, literature on iron metabolism in normal and pathological conditions.

Despite much conjecture, little definite knowledge is available about the mechanism of absorption and excretion, and the mechanism for the transport and mobilization of the iron reserves of the organism. Recent reviews summarizing the present status of the subject have been published by Hahn (5) and by Heath and Patek (6).

In the present work, with radioactive iron as an indicator, the absorption, distribution, and excretion of this element were studied in normal and iron-deficient rats.<sup>1</sup>

### *Methods*

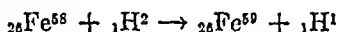
While the radioactive isotope,  $\text{Fe}^{59}$ , offers hope of throwing new light on iron metabolism, the weakness of its radiation makes its

<sup>1</sup> Expiration of Dr. Austoni's leave limited the scope of the investigation and prevented carrying out as complete a study as had been planned.

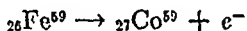
quantitative estimation quite difficult. Furthermore, the  $\beta$ -rays emitted are of a low energy and are readily absorbed (4). This difficulty is enhanced by defects in the chemico-analytical methods for the separation of iron, which are far from being satisfactory for biological material.

Having begun a study of iron metabolism with labeled iron, the authors soon became aware of the necessity of standardizing the analytical technique for separating the iron and measuring its radioactivity. The reports published by Hahn, Bale, Lawrence, and Whipple (7) furnished us with further proof of this necessity. Thus, as a first step, a survey of methods was carried out and procedures were developed which appeared adequate for the present study, although they were by no means ideal.

The radioactive iron used in this work was produced by bombardment of iron phosphide with deuterons in the cyclotron of the Radiation Laboratory of the University of California. The reaction that takes place is



The half life of  $\text{Fe}^{59}$  is  $47 \pm 3$  days, and its decay is depicted by the reaction



The iron was isolated from the iron phosphide by the procedure described by Livingood and Seaborg (4). In most cases, the iron was extracted as ferric chloride from HCl solution with ether, but, in several instances, recourse was had to the alternative procedure of precipitating the iron as ferric hydroxide. The purity of the different samples was established by determining the decay curves.

The estimation of the radioactivity was carried out mainly with a Lauritsen electroscope, which proved sensitive enough for most purposes. Samples too weak for the electroscope were measured with an ionization electrometer (8). The experimental results are expressed either in arbitrary electroscope units,<sup>2</sup> or in terms of the per cent of total radioactive iron administered.

*Absorption of Radiation and Recovery of Iron*—The experiments reported below were carried out in order to determine the most

<sup>2</sup> 1 unit is equivalent to the background discharge of the instrument, and is approximately equal to 1/1600 microcurie.

suitable procedures for isolating the iron samples and measuring their radioactivity.

Hahn and coworkers (7) carried out the majority of their activity measurements on solutions of their samples, using the method devised by Bale, Haven, and LeFevre (9). A test, the results of which are given in Table I, showed that this is an unsuitable procedure when the measurement is made with an electro-scope upon the sample contained in a Coors ashing capsule. In solution, as would be expected upon *a priori* grounds, the degree of  $\beta$ -ray absorption is enormous. A reduction in the already low degree of radiation of the iron samples would greatly enhance the errors of the estimation.

Because of the soft  $\beta$ -rays emitted by iron, it appeared reasonable to suspect a considerable self-absorption by the inert iron in a radioactive preparation. The effect of inert iron and of certain

TABLE I  
*Absorption of  $\beta$ -Rays from  $Fe^{59}$  in Solution*

	Dry $Fe_2O_3$ (1.66 mg. Fe)	Fe dissolved in 1 N $H_2SO_4$				
		1 ml.	2 ml.	3 ml.	5 ml.	8 ml.
Radioactivity, units.....	210	60	47	20	14	9.6
Absorption, per cent.....	0	71.5	77.7	86.2	93.5	95.5

combined anions was determined by adding varying amounts of  $FeCl_3$  to a sample of radioactive  $FeCl_3$  and measuring the radiation of the iron in the form of the chloride, hematite, magnetite, and sulfate.

From the results obtained, which are given in Table II, it appears that self-absorption by the inert iron can be of little consequence. Additions of far larger quantities of iron than were present in the initial labeled iron sample produced no measurable reduction in the radioactivity. On the contrary, the absorption of the anions seems quite important, the amount being dependent on the quantity of anion present. This test established the fact that conversion of iron to its oxides would yield a suitable medium for measuring the radioactivity of labeled samples.

The degree to which the radiation of  $Fe^{59}$  is absorbed by the inorganic matter present in biological material was tested on

artificial salt mixtures, and on the ash of rat tissues to which a measured quantity of radioactive iron was added. Aliquot portions were evaporated in Coors ashing capsules, heated, and the

TABLE II  
*Test of Absorption of Radioactivity by Iron and Iron Salts*

Quantity of Fe*	Radioactivity units; measured as				
	FeCl <sub>3</sub> , dried at 100°	Fe <sub>2</sub> O <sub>3</sub> , heated to 360°	Fe <sub>2</sub> O <sub>3</sub> + Fe <sub>3</sub> O <sub>4</sub> , heated to 700°	FeSO <sub>4</sub> + Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> †	Fe <sub>2</sub> O <sub>3</sub> + Fe <sub>3</sub> O <sub>4</sub> , heated to 750°‡
<i>mg.</i>					
0.9§	97	101	100	83	92
1.9	100	104.5	103	100	101
2.9	95	101	100	98	101
3.9	91	101	99.5	95	100.5
4.9	88	100.5	100	94	100.5
5.9	93	103.5	103	94	102

\* Inert FeCl<sub>3</sub> added to the active iron sample.

† Samples represented in the preceding column dissolved in H<sub>2</sub>SO<sub>4</sub> and evaporated to dryness.

‡ Sulfate samples converted to oxides by heating to 750°.

§ Standard.

TABLE III  
*Absorption of Radioactivity of Iron by Inorganic Salts*

Sample		Heated at 300-360°	Heated at 700-750°
	<i>ml.</i>	<i>per cent</i>	<i>per cent</i>
Salt mixture*	1	0	0
" "	20	3	3
" "	50	11	12
Tissue ash solution	2	9	14.5
" " "	10	17.5	13.5
" " "	20	22.5	27

\* The salt mixture contained K<sub>2</sub>HPO<sub>4</sub> 6.63 gm., NaH<sub>2</sub>PO<sub>4</sub> 2.56 gm., and CaCl<sub>2</sub> 0.8 gm. per 100 ml.

radioactivity measured after cooling. Illustrative results are recorded in Table III. These tests showed that absorption of the radiation assumed larger proportions as the quantity of the inert inorganic matter increased.

The absorption of the radiation obtained by salt mixtures and tissue ash convinced us that isolation of the iron is necessary to obtain satisfactory results. This is especially true for those materials that are rich in inorganic matter, such as bone, carcass, feces, and food. Tests were carried out to determine the recovery of known quantities of labeled iron precipitated as ferric oxide and

TABLE IV

*Test of Relative Recovery of Radioactive Iron by Separation As Sulfide and As Hydrozide from Body Tissues*

	Heated at 300° as Fe <sub>2</sub> O <sub>3</sub>		Heated at 700° as Fe <sub>2</sub> O <sub>3</sub>	
	Radio-activity	Recovery	Radio-activity	Recovery
	units per 100 ml.	per cent	units per 100 ml.	per cent
Standard radioactive iron.....	390		382	
25 ml. solution of tissue ash; FeS separation (I)*.....	300	76.9	316	82.7
2 mg. Fe carrier added; FeS separation (II).....	64	16.4	60.8	15.9
2 mg. Fe carrier added; FeS separation (III).....	1.2	0.3	1.2	0.3
Total recovery.....	365.2	93.6	378.0	98.9
25 ml. solution of tissue ash; Fe(OH) <sub>3</sub> separation (I).....	320	82	334	87.5
2 mg. Fe carrier added; Fe(OH) <sub>3</sub> separation (II).....	2.0	0.5	4.8	1.2
2 mg. Fe carrier added; Fe(OH) <sub>3</sub> separation (III).....	6.4	1.6	6.4	1.6
Total recovery.....	328.4	84.1	345.2	90.3

\* The roman numerals represent the number of precipitations.

as ferric sulfide according to the method of Jackson (10). Such a recovery experiment is shown in Table IV. The results indicated that the best recovery is obtained by the sulfide precipitation method, with inert iron as a carrier.

The experiments carried out to test the absorption and recovery of administered samples of iron led us to adopt the following technique of operation for this work.

Because of its greater convenience, the organic matter of the samples was removed by dry ashing. Jackson (10) has pointed out that there is a possibility of loss of iron in the form of ferric chloride by dry ashing, especially in the presence of large amounts of potassium and sodium chloride. The possibility of such loss was minimized by carrying out the early heating at a low temperature (350–400°) and increasing it not to exceed 700°. An experimental test of this procedure with labeled iron showed that, if the early heating is carried out at low temperature, the iron can then be strongly heated without any loss.

After ashing, the samples were dissolved in hot 6 N HCl. For isolation of the iron, aliquots of one-tenth or one-twentieth of the sample of bone, feces, or carcass were taken and the total dissolved ash for the other tissues. The pH was then adjusted with ammonia to the turning point of methyl red, and the iron was precipitated as the sulfide with  $\text{H}_2\text{S}$ . This procedure is recommended by Jackson as a method of separating iron in the presence of phosphate. In the presence of large amounts of calcium phosphate, such as occur in bone, carcass, and feces, a precipitate, probably of iron and calcium phosphate, came down during the neutralization. This was not large in quantity and was filtered off along with the precipitated iron sulfide. Because of the finely divided nature of the precipitate, the iron sulfide was filtered through a sintered glass filter covered with a layer of asbestos.

After the first precipitation, a known amount of iron (1 to 2 mg.) was added to the filtrate to act as a carrier, and the iron was again separated by passing  $\text{H}_2\text{S}$  through. On materials from which iron is difficult to separate, this operation was repeated a second time. Lastly, the collected iron precipitates were dissolved with hot 6 N HCl, and the total or aliquot portions were evaporated to dryness and heated to convert the iron to the oxide. The radioactivity was measured in this form.

The total radioactive iron recovered from each animal was computed by adding the values for excreta and carcass to those for blood and tissues. Recovery was ordinarily 80 to 90 per cent of the iron administered.

### *Plan of Experiments*

Experiments were carried out by administering radioactive iron to two groups of rats, normal and iron-deficient. The normal

animals were reared on our stock colony diet, while the other group was maintained on an iron-deficient diet composed largely of dried skim milk.<sup>3</sup>

Hemoglobin values for each animal were determined at weekly intervals, with a Newcomer hemoglobinometer. At the time of administering the radioactive iron, the hemoglobin values for the normal rats ranged from 14.4 to 16.1 gm. per 100 ml. (85 to 95 per cent) and for the anemic rats from 5.9 to 9.3 gm. per 100 ml. (35 to 55 per cent).

The radioactive iron, in solution as ferric chloride, was administered to the rats by stomach tube in doses of 2 to 8 mg. The size of the dose was governed by the weight of the rat and the radioactive strength of the sample.

The animals were then placed in individual metabolism cages over a device permitting separate collection of urine and feces, and were fed the stock colony diet.

A few of the normal animals were given radioactive ferrous sulfate instead of ferric chloride, but no significant difference in absorption was observed.

After the desired period had elapsed, the rat was anesthetized, a blood sample was drawn, and the animal was viviperfused by the technique of Austoni, Rabinovitch, and Greenberg (11). The blood and desired tissues were separated and analyzed for their radioactive iron content. The radioactive iron in urine and feces was also determined.

#### EXPERIMENTAL

*Absorption*—The passage of the administered doses of radioactive iron along the gastrointestinal tract is shown in Fig. 1, in which the data represent the labeled iron of the viscera and their contents. Removal of iron from the stomach and small intestine required about 24 hours. The stomach showed a regular decrement in the radioactive iron with time. The peak in labeled iron concentration in the small intestine was obtained in the first sampling period (3 hours). How much earlier the peak may have appeared cannot be stated. Labeled iron was present in the large intestine very soon after its administration, considerable quantities appearing as early as 3 hours.

<sup>3</sup> 84 per cent powdered skim milk (Klim), 15 per cent Crisco, and 1 per cent cod liver oil. 1 mg. of crystalline thiamine chloride was added per 100 gm. of diet. The Fe content was from 4 to 7 mg. per kilo of food.



In the large intestine, the level of labeled iron reached a maximum, and then decreased with time. This maximum was lower in the anemic rats, and occurred later than in the normal rats.

The curves of Fig. 1 show that the passage of iron through the gastrointestinal tract is slower in the anemic animals. This

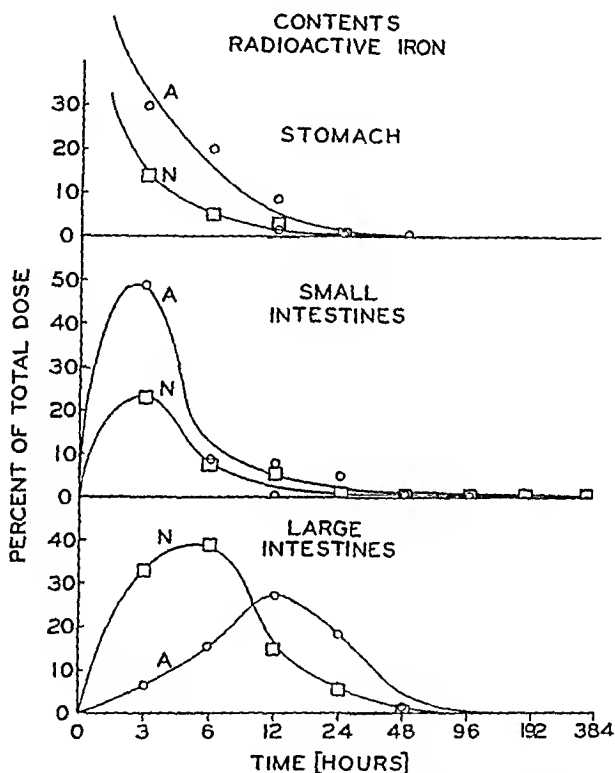


FIG. 1. Rate of passage of radioactive iron through the gastrointestinal tract of normal and anemic rats. The curves of control animals are marked *N* and the points by squares; the curves of anemic animals are marked *A* and the points by circles.

probably is the result of a hypotonicity of the gastrointestinal tract in the anemic animals.

The anemic animals absorbed a larger fraction of the administered iron. This has already been observed by Hahn and co-workers (7) on dogs, and might have been expected from the available knowledge on iron metabolism (5, 6). The possibility

exists that the delay in the passage of the iron through the gastrointestinal tract may be a factor in the increased absorption.

The time relationship found for the disappearance of iron from the stomach and small intestine agrees well with the absorption of iron deduced from the curves of serum iron obtained by Moore and coworkers (12).

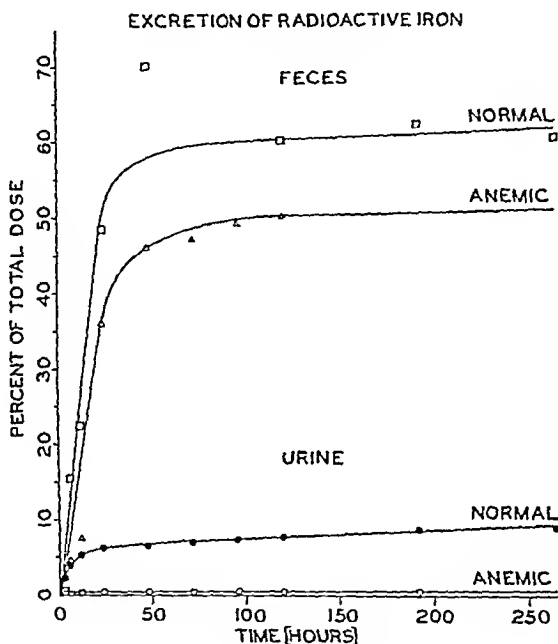


FIG. 2. Curves of the elimination of radioactive iron in feces and urine by normal and anemic rats.

*Excretion*—The curves of the excretion of labeled iron in the feces and urine are plotted in Fig. 2. The more complete utilization of the orally administered iron by the anemic rats is determined both by a greater degree of absorption and by a lessened degree of urinary excretion. The normal animals lost more iron both in the feces and in the urine. The bulk of the excretion occurred during the first 24 hours, and dropped to a small value thereafter.

Over a 10 day period the normal animals lost about 60 per cent

and anemic animals about 50 per cent of the administered iron in the feces. The urinary excretion in the same period was about 10 per cent for the normal and only about 1 per cent for the anemic animals.

*Distribution*—In an evaluation of the relative importance of the different body tissues for the uptake and storage of ingested iron,

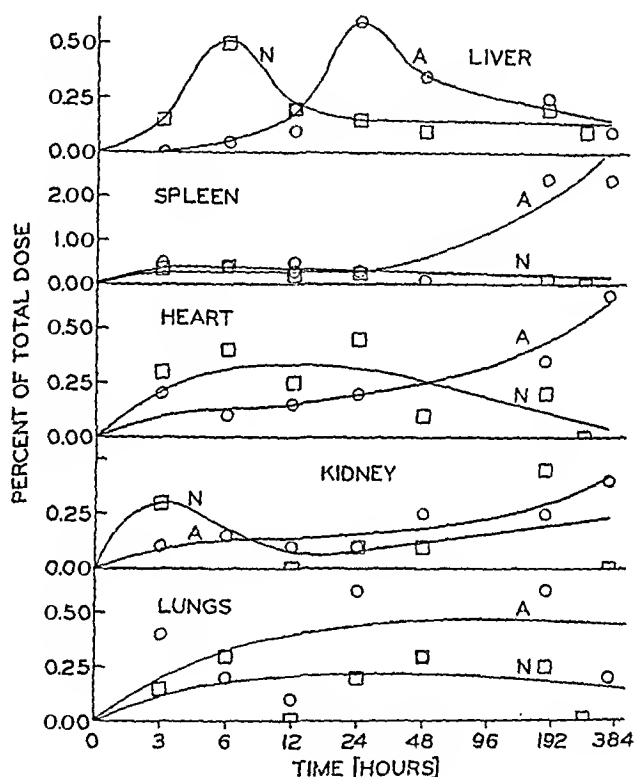


FIG. 3. Specific accumulation of radioactive iron by certain viscera of normal and anemic rats. Specific accumulation is represented in terms of per cent of the administered dose per gm. of fresh tissue.

a distinction must be made between those tissues which show a high specific accumulation per gm. of tissue, but which are small in size, and those tissues with a low specific accumulation, but which represent a large fraction of the mass of the body.

The curves for the specific accumulation of the major viscera and tissues of the body, in terms of the uptake of administered iron in per cent of dose administered per gm. of fresh tissue, are plotted

in Figs. 3 and 4. They fall into three groups according to the order of magnitude of the specific accumulation. The highest specific accumulation occurred in bone marrow, blood, and spleen. An intermediate group is represented by the liver, heart, kidney, and lungs. Lowest values were obtained for the brain and, in normal animals, for bone and muscle.

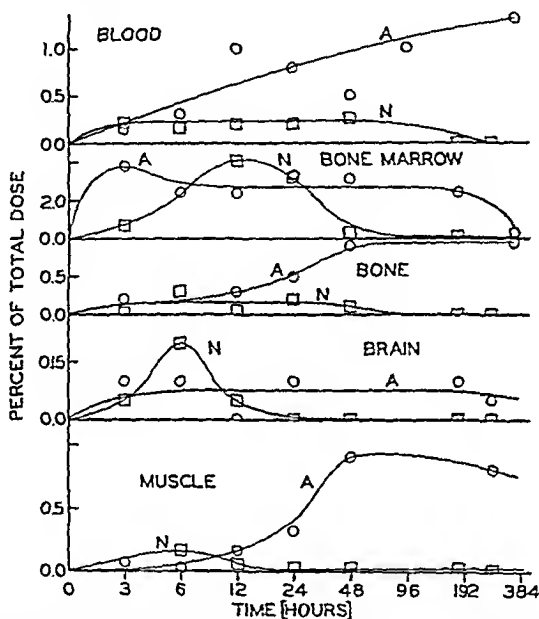


FIG. 4. Specific accumulation of radioactive iron by certain tissues of normal and anemic rats. Specific accumulation is represented in terms of per cent of the administered dose per gm. of fresh tissue.

Although they have a high specific accumulation, the spleen and the liver account for but a small fraction of the absorbed iron even in the anemic animals. On the other hand, muscle, despite the low specific accumulation, accounts for a considerable quantity of the absorbed iron.

Figs. 3 and 4 show that the lag in the rate of absorption of iron in the anemic rats, deduced from the change in the gastrointestinal contents, is reflected in the curves of accumulation of most of the tissues.

Curves of the total accumulation of labeled iron are given in Fig. 5 for certain tissues which play an important rôle in iron storage. The values for total muscle and blood in the body were calculated from the percentage amounts of these tissues in the rat as given by Donaldson (13). From Fig. 5 it may be seen that the blood and total muscle, particularly in anemia, store by far the

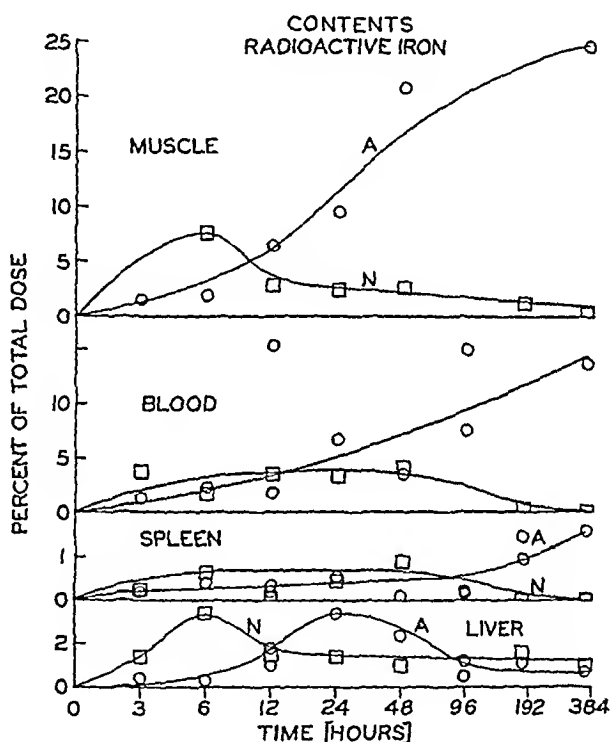


FIG. 5. Total accumulation of radioactive iron by tissues of major importance in iron metabolism in normal and anemic rats. The accumulation is represented in terms of per cent content of administered dose of radioactive iron.

greatest amount of the absorbed iron. In comparison, the total uptake of the spleen and liver is relatively small. The bone marrow also must be considered as an important storage site. This is indicated by its high specific accumulation value (Fig. 4). Since it is difficult to determine the total marrow content of the body, it was not possible to estimate the total amount of the

administered radioactive iron that was stored in this tissue in the experimental rats.

#### DISCUSSION

The present investigation represents the first reported study of the passage of iron through the gastrointestinal tract with radioactive iron as a tracer. Also the distribution of labeled iron was determined in a number of tissues not covered by Hahn and co-workers (7) in their published work with radioactive iron.

The results of this investigation, in general, are in agreement with the accepted views concerning iron metabolism (5, 6). The data confirm the already known importance of the bone marrow, blood, liver, and spleen for iron metabolism. They indicate an importance of the muscles for the storage of iron in anemia which has hitherto not been brought to light. Fig. 5 shows that, after 10 days, about 25 per cent of the administered iron had accumulated in the muscles. This represents about half of the iron absorbed. Some, but not much, of the iron in the muscles may be due to contained blood, as the muscles are difficult to perfuse thoroughly. Hahn and Whipple (14), although they were able to account for only half of the intravenously injected colloidal iron hydroxide in the perfused tissues of an anemic dog, could find no storage of iron in the muscles. The colloidal iron was administered over a 2 day period, and the dog was sacrificed and perfused about a day later. It should be noted that this interval of time just preceded the increase in the labeled iron of muscles of the anemic rats. The discrepancy in the findings points to the need of a further study of the storage of iron by muscles.

The significant conclusion of Hahn and coworkers (7) that, "The need of the body for iron in some manner determines the absorption of this element," has been confirmed by the greater absorption of labeled iron observed in anemic rats. As has already been pointed out, the slower passage of iron through the gastrointestinal tract of the anemic animals may be a factor in the greater absorption.

We are indebted to the generosity of Professor E. O. Lawrence and Dr. M. D. Kamen of the Radiation Laboratory of the University of California for the radioactive iron used in these experiments.

Technical assistance was furnished by the personnel of the Works Progress Administration (Official Project No. 65-1-08-62).

#### SUMMARY

1. With radioactive iron as an indicator, the absorption, distribution, and excretion of this element were studied in normal and iron-deficient rats.

2. It required about 12 hours for a single dose of iron to pass from the stomach and small intestine. Passage through the gastrointestinal tract was significantly slower in the anemic rats.

3. The anemic animals eliminated less of the administered iron in both feces and urine. During a 10 day period, the normal animals retained about 30 per cent of the administered iron, while the anemic animals retained 50 per cent. The greater part of the elimination through the feces and urine took place within 48 hours.

4. The specific accumulation of the absorbed iron per gm. of tissue was greatest in the bone marrow, blood, spleen, liver, and heart. The total accumulation was greatest in muscle and blood, particularly in anemic rats. After 10 days, the radioactive iron had nearly disappeared from the muscle and blood of the normal rats, but showed marked accumulation in the muscle (25 per cent of administered dose) and blood (14 per cent of administered dose) of the anemic animals.

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# THE OXIDATION OF *l*(-)-ASPARTIC AND *l*(+)-GLUTAMIC ACIDS BY *HEMOPHILUS PARAINFLUENZAE*

## NOTE ON THE PREPARATION OF PYRIDINE NUCLEOTIDES FROM BAKERS' YEAST BY THE METHOD OF WARBURG AND CHRISTIAN

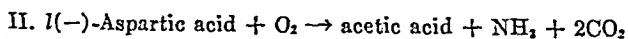
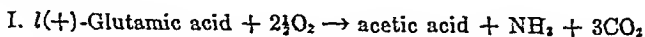
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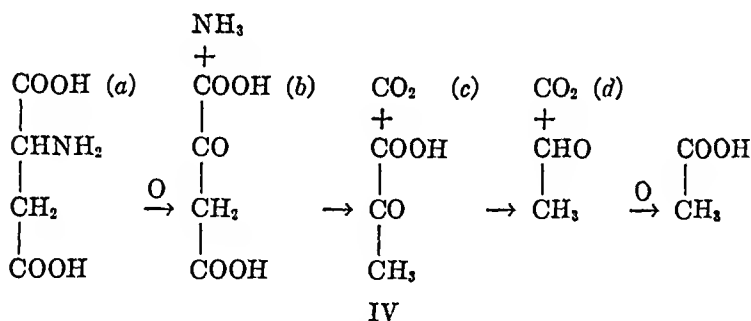
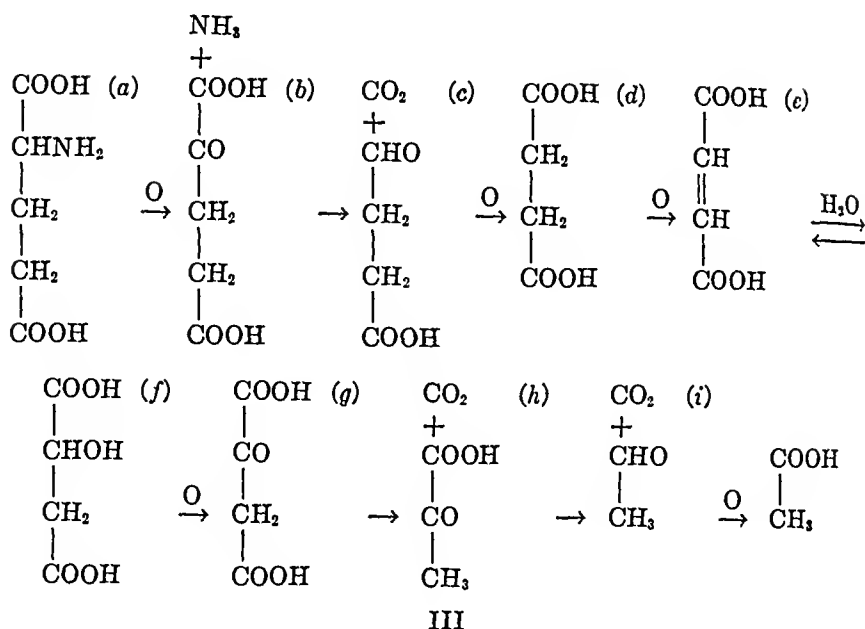
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The organism, *Hemophilus parainfluenzae*, requires factor V as an essential nutrient (1). It has been shown that di- and triphosphopyridine nucleotides can serve as factor V (2). When the bacteria have utilized the available factor V, growth and the ability to perform those functions requiring pyridine nucleotide cease. If pyridine nucleotide is added to the bacteria, their ability to grow and carry out various reactions is restored. Either nucleotide can be added, since they are interconvertible in the bacteria (2).

In the present work the oxidation of amino acids by *Hemophilus parainfluenzae* has been studied. It was found that *l*(+)-glutamic and *l*(-)-aspartic acids were oxidized, and that the oxidations required pyridine nucleotide. An attempt to determine which nucleotide was required in the oxidations was not successful. Data were obtained which indicate that *l*(+)-glutamic and *l*(-)-aspartic acids were oxidized according to Equations I and II respectively.



An analysis was made of the steps in the oxidations of the amino acids to acetic acid. Data were obtained which suggest that the reactions may proceed through the intermediates indicated in the series of Equations III and IV.



The series of reactions postulated is based upon the following observations. The oxidations of the substrates indicated in steps (d), (e), (f), (h), and (i), Equation III, and those indicated in steps (c) and (d), Equation IV, proceed with an uptake of oxygen and carbon dioxide production in agreement with the equations. Acetic acid was detected as at least one end-product of the oxidation of each substrate. Steps (a), (f), and (i), Equation III, and steps (a) and (d), Equation IV, require pyridine nucleotide. Alanine was not oxidized. Therefore, the oxidation of *l*(-)-aspartic acid could not proceed through alanine; *i.e.*, by decarboxylation at carbon atom 4. Decarboxylation of *l*(+)-glutamic acid at carbon atom 5 to form  $\alpha$ -aminobutyric acid is unlikely, since no other amino acid was oxidized. Decarboxylation of the

half aldehyde of succinic acid at carbon atom 4 and oxidation of the aldehyde to propionic acid or the formation of propionic acid from succinic acid is precluded, since propionic acid was not oxidized. Decarboxylation of oxalacetic acid at carbon atom 1 and oxidation of the aldehyde to malonic acid are not possible, since malonic acid was not oxidized. Malonic acid, which inhibits the oxidation of succinic acid (3), also inhibits the oxidation of *l*(+)-glutamic acid. Succinic acid as an intermediate in the oxidation of the amino acid therefore seems probable.

Most of the reactions postulated have been shown to occur in biological material. *l*(+)-Glutamic acid is oxidized to  $\alpha$ -ketoglutaric acid by animal tissue (4), bacteria (5), and plants (6).  $\alpha$ -Ketoglutaric acid is converted to succinic acid by animal tissue (7). Succinic acid is oxidized to fumaric acid by animal tissue (8) and bacteria (3). Fumaric acid is converted to *l*(-)-malic acid by animal tissue (8) and bacteria (9). *l*(-)-Aspartic acid is oxidized to oxalacetic acid by animal tissue (10). *l*(-)-Malic is oxidized to oxalacetic acid by animal tissue (11) and bacteria (12). Oxalacetic acid is converted to pyruvic acid by animal tissue (13) and bacteria (14). Pyruvic acid is converted to acetaldehyde by animal tissue (15) and yeast (16) and to acetic acid by animal tissue (17), bacteria (18, 19), and yeast (20). Acetaldehyde is oxidized to acetic acid by animal tissue (21) and bacteria (22). All of the oxidations, except that of succinic to fumaric acid and that of *l*(-)-aspartic to oxalacetic acid, have been shown to require pyridine nucleotide.

The present data do not exclude dismutations of the type demonstrated anaerobically in *Bacterium coli* (23, 19) as the mechanism of the oxidation of any of the substrates; e.g., pyruvic acid. The oxidation of pyruvic acid may be as demonstrated for gonococci (18), i.e.  $\text{pyruvic acid} + \frac{1}{2}\text{O}_2 \rightarrow \text{acetic acid} + \text{CO}_2$ , or through acetaldehyde as indicated in Equation III. It has not been demonstrated whether the conversion of  $\alpha$ -ketoglutaric acid to succinic acid proceeds in one or two steps.

The following amino acids were neither oxidized nor hydrolytically deaminated under the conditions of the experiments: the *dl* form of leucine, tyrosine, phenylalanine, methionine, cystine, alanine, valine, isoleucine, proline, serine; the *l* form of histidine, diiodotyrosine, tryptophane, dihydroxyphenylalanine; *d*(+)-as-

partic acid; and glycine. It has been stated that the bacteria will oxidize *dl*-valine (2).

### *Methods and Material*

The bacteria employed were from a subculture of *Hemophilus parainfluenzae*, Strain 4101, National Collection of Type Cultures, Lister Institute, London. The stock cultures were grown in a medium containing per 100 ml. 2 gm. of proteose-peptone (Difco), 0.6 gm. of sodium chloride, 0.1 gm. of sucrose, 0.04 gm. of fumaric acid, and sufficient sodium hydroxide solution to produce a pH of 7.8. Factor V, prepared from yeast, was added in excess (24).

If no factor V is added to the medium, no growth will occur. When small amounts are added, the growth is proportional to the amount added (24, 25), and at the end of the growth period the cells are deficient in factor V (2).

For the experiments described below 1 ml. of 24 hour stock culture was added to 200 ml. of medium containing 1 ml. of factor V preparation, representing the hot aqueous extract of approximately  $5 \times 10^{-3}$  gm. of bakers' yeast. After incubation at 37° for 12 to 20 hours, the bacteria were centrifuged out of the medium. They were suspended in 25 ml. of a solution of 0.9 gm. of sodium chloride per 100 ml., and again centrifuged. They were then suspended in 20 ml. of Ringer-phosphate solution, pH 7.4. 1.5 ml. of this suspension were used in each vessel.

Oxygen uptake and carbon dioxide production were measured manometrically in the usual Warburg apparatus. At the end of the manometric measurements 0.2 ml. of 20 gm. per 100 ml. trichloroacetic acid was added. The bacteria were removed by filtration. Ammonia was determined by nesslerizing an aliquot of the filtrate. Acetic acid was detected by the use of the lanthanum test (26). The concentration of acetic acid in a typical experiment was too low to be detected directly. The contents of several vessels were combined, and the bacteria removed by centrifugation. The fluid was made alkaline with sodium hydroxide solution and evaporated to a volume of 0.5 ml. The reagents were added in the proportions described. Under these conditions the test for acetic acid was obtained. It was quantitatively determined by titrating the steam-volatile acids with standard alkali.

The pyridine nucleotides were prepared from bakers' yeast by

a combination of the methods of Warburg and Christian. The purity of the diphosphopyridine nucleotide was 42 per cent (of the triphosphopyridine nucleotide 10 per cent) as estimated from their nicotinic acid content. The method of preparation is described in the "Appendix."

#### EXPERIMENTAL

In Table I are given typical data obtained with each of the substrates studied. The ratio, *moles of oxygen used to moles of substrate employed*, is a measure of the extent of the reactions. The agreement of the observed values with those predicted by Equations I to IV is probably within the experimental error. If the apparent incomplete oxidations are real, the reactions postulated account for at least the greater part of the substrate used. The observed ratios, *moles of oxygen used to moles of carbon dioxide produced to moles of ammonia produced*, are likewise in reasonably good agreement with those calculated from Equations I to IV.

Acetic acid was detected by the lanthanum test in the case of each of the substrates employed. Acetic acid was not oxidized by the bacteria. Propionic acid, which also gives the lanthanum test (26), was not oxidized.

In Figs. 1 to 3 are shown the rates of oxidation of some of the substances studied. For comparison the rate of oxidation of glucose is included in Fig. 1. The magnitude of the control respiration is also shown.

With some preparations of bacteria the rates of oxidation were independent of the concentration of the substrate (*cf.* Fig. 2) and the kind of substrate (*cf.* Fig. 1). This was not invariably the case (*cf.* Fig. 3). The factors producing this difference between various preparations were not determined.

It is stated that the rate of methylene blue reduction is much greater in the presence of glucose than in the case of other substrates (2). In the present experiments the rate of oxidation of the various substrates was of the same order as glucose.

*Oxidation of l(-)-Aspartic Acid*—The data in Table I show that the oxidation of l(-)-aspartic acid proceeds with an oxygen uptake and carbon dioxide and ammonia production in agreement with Equation II.

For a quantitative estimation of acetic acid production 15 ml.

TABLE I

*Oxidation of l(-)-Aspartic, l(+)-Glutamic, Succinic, Fumaric, dl-Malic, and Pyruvic Acids and Acetaldehyde by Hemophilus parainfluenzae*

Each vessel contained 1.5 ml. of a suspension of bacteria in Ringer-phosphate solution, pH 7.4; 0.2 ml. of a solution of pyridine nucleotide (containing 0.2 mg. of triphosphopyridine preparation or 0.05 mg. of diphosphopyridine nucleotide preparation) or Ringer-phosphate solution. The bacteria in each vessel represent one-thirteenth of the amount obtained from 200 ml. of medium. The side arm contained the substrate dissolved in 0.3 ml. of Ringer-phosphate solution. The temperature was 37.5°. The vessels were filled with air. The duration of the experiments was from 1.5 to 4 hours. The appropriate control values have been subtracted. Except where indicated, the oxidation did not occur unless pyridine nucleotide was added. The oxidations occur with either nucleotide.

Substrate	Amount added in terms of one isomer	O <sub>2</sub> uptake	CO <sub>2</sub> produced	NH <sub>3</sub> produced	Ratio, mm of O <sub>2</sub> uptake to mm of substrate used		Ratio, mm of O <sub>2</sub> uptake (moles oxygen = 1) to mm of CO <sub>2</sub> produced to mm of NH <sub>3</sub> produced	
					Observed	Calculated from Equations I-IV	Observed	Calculated from Equations I-IV
l(-)-Aspartic acid	0.0038	0.0036	0.0062	0.0036	0.84	1.0	1:1.94:1.12	1:2:1
	0.0015	0.0013	0.0027	0.0014	0.87	1.0	1:2.08:1.07	1:2:1
dl-Aspartic acid	0.0038	0.0041			1.08	1.0		
l(+)-Glutamic acid	0.0034	0.0084	0.0100	0.0030	2.47	2.5	1:1.19:0.40	1:1.2:0.4
	0.0014	0.0034	0.0039	0.0013	2.43	2.5	1:1.15:0.39	1:1.2:0.4
Succinic acid, no added nucleotide	0.0042	0.0020	0.0000		0.48	0.5	1:0	1:0
Succinic acid	0.0042	0.0069	0.0085		1.64	1.5	1:1.23	1:1.33
	0.0021	0.0035	0.0042		1.66	1.5	1:1.20	1:1.33
Fumaric "	0.0043	0.0033	0.0069		0.77	1.0	1:2.09	1:2
	0.0043	0.0034	0.0069		0.79	1.0	1:2.03	1:2
dl-Malic "	0.0019	0.0015	0.0029		0.79	1.0	1:1.93	1:2
	0.0037	0.0031	0.0073		0.84	1.0	1:2.35	1:2
Pyruvic "	0.0057	0.0022	0.0048		0.39	0.5	1:2.18	1:2
	0.0048	0.0021	0.0042		0.44	0.5	1:2.00	1:2
" " no added nucleotide	0.0048	0.0008	0.0025					
Acetaldehyde	0.0060	0.0020	0.000		0.33	0.5	1:0	1:0

of the usual suspension of bacteria were incubated, with shaking at  $37.5^{\circ}$ , in the presence of 10 mg. of *l*(-)-aspartic acid. After 4 hours the bacteria were removed by centrifugation. The solution was transferred to a distilling flask, and 2 ml. of 18 N sulfuric acid added. The material was distilled and 10 ml. of distillate collected. The distillate was titrated with 0.05 N sodium hydroxide, with phenolphthalein as an indicator; 1.45 ml. were used. Therefore 0.0752 mm of aspartic acid was oxidized to 0.073 mm of steam-volatile acid. The agreement of the observed value

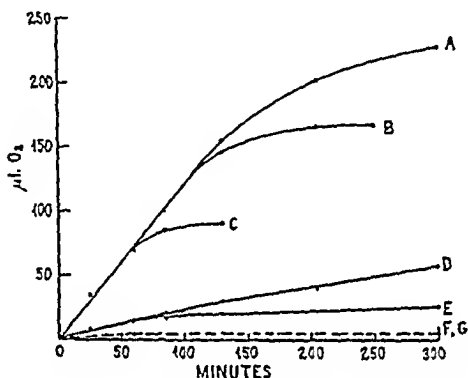


FIG. 1. The rate of oxidation of aspartic acid and glucose and the effect of added pyridine nucleotide. The plan of the experiment was as given in Table I. Curve A, 1 mg. of glucose plus 0.2 mg. of triphosphopyridine nucleotide; Curve B, 0.5 mg. of aspartic acid plus 0.2 mg. of triphosphopyridine nucleotide; Curve C, 0.25 mg. of aspartic acid plus 0.2 mg. of triphosphopyridine nucleotide; Curve D, 1.0 mg. of glucose; Curve E, bacteria plus nucleotide without substrate; Curve F, bacteria without nucleotide and substrate; Curve G, bacteria plus 0.5 mg. of aspartic acid.

with that predicted by Equation II indicates the validity of the latter.

The data in Table I show that the oxygen uptake in the presence of *dl*-aspartic acid is that expected if one isomer is oxidized. Since the *l* isomer is oxidized, it must be concluded that the *d* form is not. None of the other amino acids tried was oxidized. This indicates that the bacteria do not contain the *d*-amino acid oxidase or that the amino acids cannot penetrate the cells. It has been shown that *Bacillus proteus* and *Bacillus pyocyaneus* can



oxidize certain *d*-amino acids, but not others (27, 28). This indicates that the failure to oxidize a given amino acid may not be due to a lack of the necessary system.

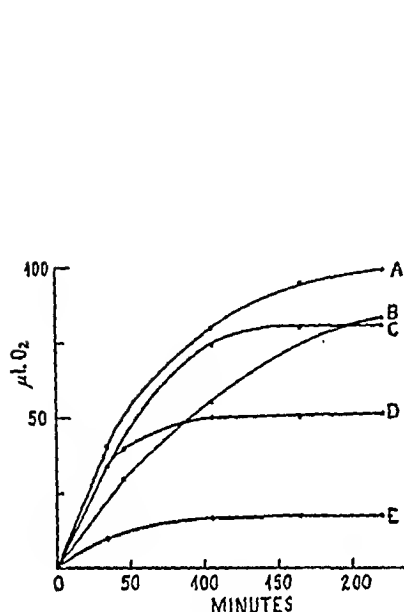


FIG. 2

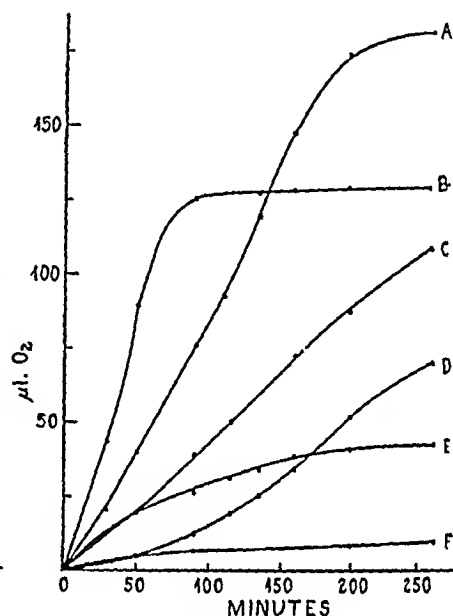


FIG. 3

FIG. 2. The rate of oxidation of *L*(-)-aspartic acid and *L*(+)-glutamic acid. The plan of the experiment was as given in Table I. The diphosphopyridine nucleotide preparation was used. Curve A, 0.2 mg. of aspartic acid plus 0.2 mg. of glutamic acid; Curve B, 0.2 mg. of glutamic acid; Curve C, 0.4 mg. of aspartic acid; Curve D, 0.2 mg. of aspartic acid; Curve E, bacteria without substrate.

FIG. 3. Inhibition of the oxidation of succinic and glutamic acids by malonic acid. The triphosphopyridine nucleotide preparation was present except when indicated. The appropriate control uptakes have been subtracted. Curve A, 0.5 mg. of *L*(+)-glutamic acid; Curve B, 0.5 mg. of succinic acid; Curve C, 0.5 mg. of succinic acid plus 0.5 mg. of malonic acid; Curve D, 0.5 mg. of glutamic acid plus 0.5 mg. of malonic acid; Curve E, 0.5 mg. of succinic acid, no nucleotide added; Curve F, same as for Curve E plus 0.5 mg. of malonic acid.

By use of the anaerobic methylene blue technique it has been shown that *Hemophilus parainfluenzae* can oxidize asparagine (2). This oxidation requires pyridine nucleotide.

*L*(-)-Aspartic acid was not oxidized by the bacteria unless

pyridine nucleotide was added. The rate of oxidation was independent of the kind of nucleotide added. This indicates that the rate of interconversion of the nucleotides is greater than the rate of oxidation of the amino acid.

*Oxidation of l(+)-Glutamic Acid*—The data in Table I indicate that the oxidation of l(+)-glutamic acid proceeds with an oxygen uptake and carbon dioxide and ammonia production in agreement with Equation I. In a large scale experiment similar to that described in the case of aspartic acid, 0.068 mm of l(+)-glutamic acid was converted by the bacteria into 0.062 mm of steam-volatile acid. These data indicate the validity of Equation I.

The bacteria did not oxidize the amino acid unless pyridine nucleotide was added. As in the case of aspartic acid the rate of oxidation was independent of the kind of nucleotide added. It has been shown that the oxidation of l(+)-glutamic acid by other bacteria requires triphosphopyridine nucleotide (5).

*Specificity of Enzyme Systems Oxidizing Aspartic and Glutamic Acids*—If two different systems are employed in the oxidation of the amino acids, summation of the rates to a certain extent might be expected when both substrates are present. In Fig. 2 data bearing upon this point are given. Fig. 2 shows that the rate of oxidation of glutamic plus aspartic acid was somewhat greater than the individual rates and that the rate of oxidation of aspartic acid was not increased by an increase in concentration of the substrate. This may indicate that two different enzyme systems are concerned in the oxidation of the two amino acids.

*Oxidation of Succinic, Fumaric, and dl-Malic Acids*—It has been shown by the methylene blue technique that the oxidation of succinic acid by the bacteria does not require pyridine nucleotide (2). As indicated by the data in Table I and Fig. 3, this applies to the oxidation of succinic to fumaric acid. Further oxidations of the fumarate require the nucleotide.

The data in Table I show that only one isomer of dl-malic acid was oxidized. Since the course of the oxidation of fumaric and of malic acid was the same, it may be suggested that the bacteria, in common with others and mammalian tissue, contain fumarase.

*Oxidation of Pyruvic Acid and Acetaldehyde*—The data in Table I show that, in the presence of added nucleotide, the greater part

of the pyruvic acid was oxidized, as suggested in Equation III or IV. The significance of the experiments without added nucleotide is not clear. The data suggest that pyruvic acid is slowly oxidized by the cells, and that carbon dioxide production is a function of that oxidation and some other factor. This reaction will be studied further.

The observed oxygen uptakes with acetaldehyde were considerably less than would be expected from Equation III or IV. Some of the substrate added probably disappears, owing to its volatility. The significant finding was that no carbon dioxide was evolved.

*Effect of Malonic Acid upon Oxidation of l(+)-Glutamic and Succinic Acids*—Malonic acid is considered a specific inhibitor of the oxidation of succinic acid (3). In Fig. 3 is shown the effect of malonic acid upon the oxidation of glutamic and succinic acids by the bacteria.

Fig. 3 shows that the oxidation of succinic acid is inhibited by malonic acid. Likewise the oxidation of glutamic acid is inhibited by malonic acid. This suggests that succinic acid is one of the intermediates in the oxidation of glutamic acid. The oxidation of l(−)-aspartic acid was not inhibited by malonic acid.

The ammonia liberated from the glutamic acid in the presence of malonic acid was approximately 20 per cent of the theoretical amount. This indicates that the mechanism of the inhibition is probably somewhat more complex than a simple inhibition of the oxidation of succinic acid. Otherwise it might be expected that all of the ammonia would be liberated, and that the succinic acid would accumulate.

#### SUMMARY

The oxidation of amino acids by *Hemophilus parainfluenzae* has been studied. Of those tested only l(−)-aspartic and l(+)-glutamic acids were oxidized in Ringer-phosphate solution.

l(−)-Aspartic acid was oxidized with the uptake of 1 mole of oxygen, the production of 2 moles of carbon dioxide, 1 mole of ammonia, and 1 mole of acetic acid.

l(+)-Glutamic acid was oxidized with the uptake of 2.5 moles of oxygen, the production of 3 moles of carbon dioxide, 1 mole of ammonia, and 1 mole of acetic acid.

Succinic acid, which is postulated as one of the intermediates in the oxidation of glutamic acid, was oxidized with the uptake of 1.5 moles of oxygen and the production of 2 moles of carbon dioxide.

Fumaric and malic acids, which are postulated as intermediates in the oxidation of glutamic acid, were oxidized with the uptake of 1 mole of oxygen and the production of 2 moles of carbon dioxide.

Pyruvic acid, which is postulated as an intermediate in the oxidation of both amino acids, was oxidized with the uptake of 0.5 mole of oxygen and the production of 1 mole of carbon dioxide.

Acetaldehyde, postulated as an intermediate in the oxidation of both amino acids, was oxidized with the uptake of 0.5 mole of oxygen.

Acetic acid was detected as the end-product of the oxidation of each of the substrates tested.

Acetic, malonic, and propionic acids were not oxidized.

The oxidation of succinic and glutamic acids was inhibited by malonic acid.

The first step in the oxidation of succinic acid, which required 0.5 mole of oxygen, did not require added pyridine nucleotide. The complete oxidation of pyruvic acid required added nucleotide. This acid was partially oxidized without added nucleotide. The oxidation of all of the other substrates required pyridine nucleotide.

I am grateful to Dr. Henry Irving Kohn for providing the initial impetus necessary for this work, and for generous aid in the preparation of the bacteria. I am indebted to Mr. E. Levy and Mr. H. Sarett for the nicotinic acid analyses of the pyridine nucleotides.

#### APPENDIX

##### *Note on the Preparation of Pyridine Nucleotides from Bakers' Yeast by the Method of Warburg and Christian*

Warburg and Christian (29) found that a phenol extract of an aqueous bakers' yeast preparation contained flavin-adenine dinucleotide and the pyridine nucleotides. The preparation of the flavin-adenine dinucleotide was described. The method for the preparation of the pyridine nucleotides was patented ((29) p. 161).

Since a description of the method for the preparation of the pyridine nucleotides was not available, the following procedure was evolved. It is based upon the methods described by Warburg and Christian (29, 30).

*Test for Total Pyridine Nucleotides*—Factor V assays were used as a measure of the total pyridine nucleotides present in the various fractions. The method has been given (24, 25).

*Test for Diphosphopyridine Nucleotide*—The test is based upon the fact that the oxidation of lactic acid by animal tissue requires diphosphopyridine nucleotide (31). All of the following steps were carried out with ice-cold materials or in the refrigerator.

A rat liver was ground with sand and 70 ml. of 0.01 M disodium hydrogen phosphate solution. The mixture was squeezed through muslin. 70 ml. of saturated ammonium sulfate were added. After the protein had begun to flocculate, the mixture was centrifuged at 4000 to 5000 R.P.M. The precipitate was suspended in 50 ml. of 0.01 M disodium hydrogen phosphate solution; 25 ml. of saturated ammonium sulfate were added. 0.2 N hydrochloric acid was added slowly with shaking until the pH was approximately 3 (Congo red paper). The mixture was centrifuged. The precipitate was suspended in 10 ml. of 0.04 M disodium hydrogen phosphate solution. Enough 0.2 M sodium hydroxide solution was added to bring the pH to approximately 7.4. This material did not oxidize lactic acid. When flavin-adenine dinucleotide and diphosphopyridine nucleotide were added, the preparation oxidized lactic acid. The data in Table II illustrate the test.

*Separation of Flavin-Adenine Dinucleotide and Pyridine Nucleotides*—A hot aqueous extract of 20 pounds of bakers' yeast (Fleischmann's) was prepared. It was extracted with liquid phenol. The flavin and pyridine nucleotides were extracted from the phenol fraction with water. The flavin-adenine dinucleotide was precipitated in acid solution as the silver salt. All of the above steps were carried out as described by Warburg and Christian (29). The filtrate obtained after the removal of the silver salt of the flavin contains the pyridine nucleotides.

To the filtrate were added 100 ml. of 20 gm. per 100 ml. mercuric acetate solution. Solid barium hydroxide was added with vigorous stirring until the mixture was just acid to litmus paper. The mixture was let stand in the refrigerator until the

precipitate had begun to settle. The precipitate was collected by centrifugation. It was washed twice with 20 ml. portions of 0.5 gm. per 100 ml. mercuric acetate solution. The precipitate was suspended in 200 ml. of water. Hydrogen sulfide was passed through the mixture for 1 hour. The mixture was centrifuged and the supernatant liquid removed. The precipitate was suspended in 100 ml. of water, treated with hydrogen sulfide, and the supernatant fluid collected. This treatment was repeated twice more. The combined supernatant liquids had a volume of about 500 ml.

TABLE II

*Effect of Addition of Flavin and Pyridine Nucleotide upon Oxidation of Lactate by Rat Liver Preparation*

The oxygen uptakes were measured manometrically at 37.5°. The vessels were filled with air. The insets contained alkali. 0.2 ml. of the flavin preparation contained approximately 200  $\gamma$  of the nucleotide; 0.2 ml. of the diphosphopyridine nucleotide preparation contained approximately 100  $\gamma$  of the nucleotide; 0.4 ml. of the lactate solution contained 4 mg. of lithium *DL*-lactate. The triphosphopyridine preparation in equivalent quantities was not active.

Liver preparation, ml.....	1.0	1.0	1.0	1.0
Flavin " " .....		0.2		0.2
Pyridine nucleotide preparation, ml.....			0.2	0.2
Phosphate buffer, 0.04 M, pH 7.4, ml.....	0.6	0.4	0.4	0.2
Lactate solution, ml.....	0.4	0.4	0.4	0.4
O <sub>2</sub> uptake, $\mu$ l. per 30 min.....	3	6	4	35

*Precipitation of Pyridine Nucleotides by Acetone*—The filtrate was concentrated *in vacuo* to about 150 ml. 5 volumes of acetone were added. The mixture was kept in the refrigerator until the precipitate had settled. The precipitate was collected by centrifugation. The liquid was concentrated *in vacuo* to about 40 ml. 5 volumes of acetone were added. The precipitate formed was collected and added to the first.

Based upon the factor V assay the combined precipitates contained approximately 75 per cent of the initial pyridine nucleotides.

*Separation of Pyridine Nucleotides*—The precipitate contained about 3 gm. of pyridine nucleotides. It was dissolved in 1000 ml. of water. The solution was treated with barium acetate and hydroxide and the barium salt of triphosphopyridine nucleotide precipitated by alcohol. The separation was carried out as described by Warburg and Christian (30). The addition of the barium salts did not produce an appreciable precipitate, since the adenine nucleotides were removed by the initial precipitation with silver nitrate.

One precipitation of each nucleotide with mercuric acetate and one with acetone were carried out as described (30).

The yield was 2.5 gm. of triphosphopyridine nucleotide of 10 per cent purity, and 0.8 gm. of diphosphopyridine nucleotide of 42 per cent purity. The purity was based upon the nicotinic acid content.

The details necessary for further purification of the nucleotides have been adequately described (30).

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## THE RESPONSE OF LIPID METABOLISM TO ALTERATIONS IN NUTRITIONAL STATE

### I. THE EFFECTS OF FASTING AND CHRONIC UNDERNUTRITION UPON THE POSTABSORPTIVE LEVEL OF THE BLOOD LIPIDS\*

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Although observations dealing with the influence of nutrition upon one or other lipid constituent of the blood have repeatedly appeared, no systematic treatment in which all lipid constituents were simultaneously studied during changes in nutritional state commonly met with in the laboratory is at present available. Data concerning the maximum fluctuations that can occur in the level of the various lipid constituents of blood and liver during alterations in the caloric intake are necessary for the interpretation of lipid changes encountered; for example, in endocrine and vitamin disturbances. The observations so far reported are not consistent, owing probably to variations in the lipid methods employed as well as in the animal species studied (1-8). Bloor observed increases in the lipid level during fasting amounting to as much as 22 to 29 per cent of the initial value in three of six dogs studied. The occurrence of this rise was believed to depend upon the presence of adequate body fat. Greene and Summers reported that a lipemia occurs in younger dogs during fasting but not in older animals. Terroine investigated a group of seven dogs during a fast that extended over a period of 22 to 35 days and reported blood lipid changes ranging from a 45 per cent increase to a 54 per cent decrease. In two dogs fasted for 7 days, Ling found a

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small decrease in some of the lipid constituents of the blood. An early rise in the level of blood cholesterol in man during fasting was claimed by Shope. According to Man and Gildea, malnutrition in man is associated with a lowered lipid level of the blood. Page *et al.* found no change in the level of serum lipids of dogs subjected to a very low protein intake for long periods of time.

The present paper presents the effects of complete exclusion or partial reduction in caloric intake (*i.e.* chronic undernutrition) upon the lipid constituents of dog blood, namely cholesterol, phospholipids, and total fatty acids, as measured by oxidative procedures.

#### EXPERIMENTAL

Twenty-five adult dogs were employed in the present study, the data of eight of which are recorded here. Before fasting or reduction in the caloric intake, each animal received a diet adequate in calories, proteins, vitamins, and salts. They were fed twice daily (at 8.00 a.m. and 4.00 p.m.) a mixture of meat and sucrose. Vitamins A and D were supplied as cod liver oil<sup>1</sup> and the B complex in the form of a concentrate prepared from rice bran.<sup>2</sup> Each animal also received daily Cowgill's salt mixture (9) along with the meat and sucrose fed at 4.00 p.m.

Following this control period the animals either were fasted or had their caloric intake severely reduced. This consisted in a complete or partial elimination of the meat and sucrose constituents of the diet; the feeding of vitamin supplements and salts was not interfered with. The daily administration of cod liver oil, rice bran concentrate, and salt mixture in amounts recorded in Tables I and II was maintained throughout the period of partial or complete fasting.

Whole blood was used for lipid analyses. In animals that were fed, blood was taken just before the morning meal. Thus at the time blood was sampled, all dogs were in the postabsorptive state, having ingested their last meal 16 hours before the removal of blood. In the case of animals subjected to fasting, blood samples were removed between 8.00 and 9.00 a.m.

<sup>1</sup> The standardized cod liver oil was generously furnished by Mead Johnson and Company.

<sup>2</sup> The vitamin B concentrate was kindly furnished by Vitab Products, Inc., Emeryville, California.

Microoxidative procedures were employed for lipid determination. These have been described elsewhere (10).

### *Chronic Undernutrition*

The blood lipid changes associated with weight loss produced by reduction in the caloric intake are shown in Table I. In the seven dogs investigated (only three of which are recorded here) observations were extended over a period of 20 to 23 weeks, during which blood was removed for analyses at various intervals. In all cases the caloric intake during the control period was sufficient to maintain the initial weight of the dog. To induce chronic undernutrition, the caloric intake of all dogs was finally cut to 25 gm. of lean meat twice daily. This treatment produced severe losses in weight. At the end of the 20 to 23 weeks of observation, four of the animals (Dogs N-7, N-9, N-21, and N-22) had lost 50 per cent or more of their body weight, whereas in the remainder (Dogs N-19, N-20, and N-8) losses of 41, 34, and 17 per cent had occurred.

*Total Lipid*—A significant drop in the level of total lipids occurred, but the extent of the drop varied in the seven dogs. The most pronounced change was observed in Dog N-19. Values for total lipid taken at 2 and 4.5 weeks after this dog was placed on the control diet were respectively 731 and 708 mg. per 100 cc. of whole blood, whereas 16.5 weeks after receiving a diet of only 50 gm. of lean meat daily along with a salt mixture and vitamin supplements the level of total lipid in the blood had fallen to 488 mg. At the same time the animal's weight fell from 16.3 to 9.6 kilos, a loss of approximately 40 per cent. Striking reductions in the concentration of total lipids were also found in Dogs N-20, N-22, N-7, N-8, and N-9. Despite the loss of 50 per cent of body weight, total lipids in Dog N-21 fell from 631–582 mg. to 506 mg.

*Cholesterol*—In six of the seven dogs examined, total cholesterol fell as a result of chronic undernutrition. Although the values found at the end were lower than those observed during the control period, the onset of the change was gradual in most cases, severe decreases not being apparent till several weeks after chronic undernutrition had been maintained. The irregularity in these changes is shown by the fact that in Dog N-9 a significant drop in cholesterol first appeared in 16.4 weeks, while in Dog N-19 a decrease had occurred during the first few weeks after severe

TABLE I

*Effects of Chronic Undernutrition upon Whole Blood Lipids*

Dog No.	Period of observation							Weight	Total cholesterol	Total fatty acids	Phospho-lipid	Total lipid
	Total time	Control			Undernutrition							
		Time	Diet*		Time	Diet*						
			Meat	Su-crose		Meat	Su-crose					
	wks.	wks.	gm.	gm.	wks.	gm.	gm.	kg.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
N-7, ♀	4	4	143	28				9.5	182	372	344	554
	7				3	35	20	9.2	133	352	292	485
	9				5	35	20	8.7	127	260	255	387
	10				6	25		8.7	128	324	288	452
	11.7				7.7	25		7.4	125	337	308	462
	13.7				9.7	25		7.1	132	306	308	438
	16.6				12.6	25		6.4	128	324	288	452
	20.1				16.1†	25		5.1	134	320	286	454
	22.6				18.6†	25		4.5	130	240	225	370
N-19, ♀	2	2	215	42				15.5	233	498	438	731
	4.5	4.5	215	42				16.3	231	477	402	708
	5.5				1	25		15.4	168	432	360	600
	6.5				2	25		14.5	144	392	348	536
	8				3.5	25		14.0	162	334	335	496
	9				4.5	25		13.5	154	395	312	549
	11.5				7	25		13.3	146	412	330	558
	15.5				11	25		11.5	150	362	349	512
	18.5				14†	25		11.3	160	386	356	546
	21				16.5†	25		9.6	150	338	361	488
N-22, ♀	3	3	175	36				10.3	145	388	348	533
	4	4	175	36				10.3	158	390	360	548
	4.5	4.5	175	36				10.3	154	373	365	527
	5.5				1	25		9.8	120	326	304	446
	6.5				2	25		9.4	128	359	304	487
	8				3.5	25		8.7	154	330	308	484
	9				4.5	25		8.4	147	388	351	535
	11.5				7	25		7.5	135	393	295	528
	15.5				11	25		6.8	138	352	347	490
	18.5				14†	25		6.0	138	337	325	475
21				17†	25		5.2	100	244	221	344	

\* The amounts recorded were fed twice daily at 8.00 a.m. and at 4.00 p.m. In addition each animal received daily both during the control period and period of undernutrition 3 cc. of cod liver oil, 5 cc. of rice bran concentrate, and 2 gm. of Cowgill's salt mixture.

† Weak and emaciated.

‡ Thin but active.

caloric reduction had been instituted. In Dog N-20 changes in cholesterol were apparent as early as the 2nd week.

*Phospholipids*—Prolonged chronic undernutrition produced a fall in the level of the phospholipids in the blood, but alterations were not regularly observed during the first several weeks after animals were placed on the lowered caloric intake. In Dogs N-21 and N-22 there was a tendency for this lipid constituent to remain close to the control level for as long as 11 to 14 weeks, even though marked reductions in weight had occurred. In Dog N-20 no fall in phospholipids was found throughout the 16.5 weeks of observation. In two dogs, Nos. N-7 and N-9, severe reductions in the phospholipid level of the blood occurred at the end of the period of observation.

*Total Fatty Acids*—Prolonged chronic undernutrition produced a fall in total fatty acids of the blood, but the extent of the decrease varied considerably in the seven dogs. The end-results were in all cases lower than the control values, which were taken at a time when the dogs were maintained upon an adequate caloric intake. Thus at intervals of 16.5 to 18.6 weeks after chronic undernutrition had been instituted, the values found for total fatty acids were 240, 336, 192, 338, 411, 366, and 244 mg. as compared with control values of 372, 482, 349, 498, 492, 453, and 388 mg. respectively. The fall in the concentration of total fatty acids was not always an early effect, and even in Dogs N-7 and N-9, in which the very low values of 240 and 192 mg. were found, the level of this lipid constituent was not markedly decreased for many weeks after the caloric intake had been severely curtailed.

### *Fasting*

Typical changes in whole blood lipids produced by fasting are shown in Table II and Figs. 1 and 2. Eighteen dogs in all were studied but the results of five are recorded here. After a suitable control period, in which lipid levels were determined while the dogs were in the normal nutritional state, they were fasted for periods varying from 4 to 30 days. Fasting was restricted to the complete withdrawal of meat and sucrose from the diet; salts and vitamin concentrates were furnished daily during the entire period of observation.

*Cholesterol*—Fasting for 4 to 9 days failed to produce significant

TABLE II

*Effect of Fasting upon Whole Blood Lipids*

Dog No.	Period of observation				Fast†	Weight	Total choles- terol	Total fatty acids	Phos- pho- lipid	Total lipid
	Total time	Control								
		Time	Diet*							
			Meat	Su- crose						
	days	days	gm.	gm.	days	kg.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
N-31, ♂	0	0	110	22		10.6	156	340	300	496
	7	7	110	22		10.8	161	338	308	499
	9				2	10.5	138	360	315	498
	10				3	10.3	138	400	337	538
	14				7	9.7	111	370	293	481
	16				9	9.6	144	324	278	468
N-To, ♂	5	5	150	40		12.2	159	413	340	572
	6	6	150	40		14.4	151	386	330	537
	7				1	14.2	136	333	257	469
	8				2	13.8	140	358	294	498
	9				3	13.6	125	361	271	486
	11				5	13.4	138	349	304	487
	13				8	12.6	125	328	355	453
	20				14	12.0	154	391	363	545
	27				21	11.0	162	398	371	560
N-No, ♀	6	6	175	25		14.8	159	371	339	530
	10	10	175	25		14.9	154	356	361	510
	12	12	175	25		14.9	161	362	346	523
	13				1	14.7	159	342	345	501
	14				2	14.5	152	326		478
	15				3	14.4	147	296	340	443
	17				5	14.3	149	358	345	507
	20				8	13.8	145	316	348	461
	24				12	12.9	137	347	363	484
	30				18	11.8	145	323	354	468

\* The amounts recorded were fed twice daily at 8.00 a.m. and at 4.00 p.m. In addition, all dogs received once daily 3 cc. of cod liver oil, 5 cc. of rice bran extract, and 2 gm. of Cowgill's salt mixture.

† During fasting all dogs received once daily 3 cc. of cod liver oil, 5 cc. of rice bran extract, and 2 gm. of Cowgill's salt mixture.

changes in the level of blood cholesterol. The level did not always remain constant in the blood during the period of observation, but when fluctuations occurred during fasting they did not differ

significantly from those noted during control periods in which the animals received adequate diets.

Even in more prolonged fasts (18 to 21 days) it is striking to find the levels of cholesterol maintained with little change through the entire period. Thus in Dog N-No, the concentration of cholesterol at the end of an 18 day fast was 145 mg. as compared with values of 154 to 161 mg. found just before the start of the fast. Similar results were obtained in Dog N-To, which was observed during a fast of 21 days while it lost 23 per cent of its body weight.

*Phospholipids*—Dogs N-31, N-To, and N-No were fasted for 9, 21, and 18 days respectively, and the values found for phospholipids at the end of the fasting period were close to those observed during the control period. In the other dogs that underwent a short fast, the end-values, taken at 4, 6, and 9 days after fasting was instituted, were only slightly below those of the control period.

*Total Fatty Acids*—No change in the level of total fatty acids was produced in Dog N-31 by a short fast of 9 days. In Dogs N-To and N-No slight changes apparently occurred as early as 8 days, but in Dog N-35 a loss of over 100 mg. was observed 6 days after complete fasting was begun.

*Observations on Repeated Fasts in Same Animal. Cholesterol*—The experimental procedures employed for Dogs N-PB and N-ST differed from those employed for the dogs shown in Table II, in that the former were observed during periods of fast that were alternated with periods in which they received maintenance diets. Thus blood lipid examinations were made during periods of weight loss and periods of weight gain. Just before the start of the fast the blood of Dog N-PB contained 156 mg. of cholesterol (A, Fig. 1), whereas 30 days later, after a period during which the animal received no calories other than those contained in the small amounts of vitamin concentrates administered, the cholesterol had fallen to a value of 130 mg. per 100 cc. of whole blood (B, Fig. 1). During the next 26 days (BC) this dog was fed 250 gm. of lean meat and 25 gm. of sucrose (the maintenance diet) twice daily, and this led to a gain of 1.6 kilos in weight. At the end of the 26 days cholesterol was present to the extent of 151 mg. (C, Fig. 1). The blood lipids of this animal were next examined after a 10 day fast (CD) and then after a 9 day feeding period, and the values



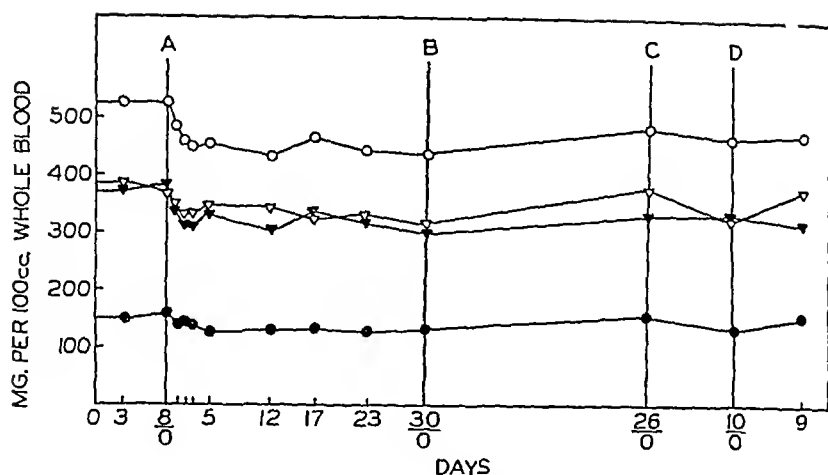


FIG. 1. The response of the blood lipids of Dog N-PB to repeated fasting. During the intervals *AB* and *CD* the animal was fasted; it was fed at the other intervals. When fed, this animal received 250 gm. of lean meat and 25 gm. of sucrose twice daily in addition to a salt mixture and vitamin concentrates. This dog weighed 20.9 kilos at 0 day, 22 kilos at *A*, 15.9 kilos at *B*, 17.5 kilos at *C*, 15.6 kilos at *D*, and 16.4 kilos at the end of the period of observation. ○ total lipid, ▼ total fatty acid, ▽ phospholipid, ● total cholesterol.

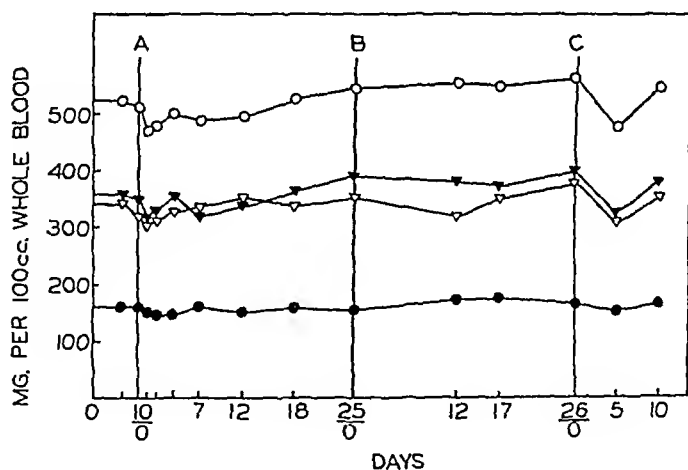


FIG. 2. The response of the blood lipids of Dog N-ST to repeated fasting. During the intervals *AB* and after *C* the animal was fasted; it was fed at the other intervals. During feeding and fasting periods this animal received the same treatment as Dog N-PB. This dog weighed 13.9 kilos at 0 day, 14 kilos at *A*, 9.9 kilos at *B*, 11.5 kilos at *C*, and 10 kilos at the end. The symbols have the same meaning as for Fig. 1.

found at the end of each of these periods again point to the relative stability in the level of cholesterol in the blood.

In Dog N-ST cholesterol determinations for four periods are recorded in Fig. 2. The values found during the control period that preceded the first fast were 159 and 161 mg. for cholesterol. During the fasting period that lasted for 25 days (AB) the blood was examined on seven occasions, and the values fluctuated between 145 and 161 mg. per 100 cc. of whole blood. During the next two periods, namely one in which the animal received for 26 days the maintenance diet (BC) and the other in which the animal fasted for 10 days (following C), the levels of cholesterol were maintained at values similar to those recorded above.

*Phospholipid*—The level of phospholipid in the blood was well maintained despite the fact that fasting continued for as long as 30 days. The absence of any striking change in the phospholipid content of the blood as a result of fasting is best shown in Dogs N-PB and N-ST. In the former the initial control values were 380 and 371 mg. During a 30 day fast (AB, Fig. 1) the values fluctuated between 312 and 344 mg. At the end of a 26 day feeding period (BC), in which this animal was fed the same diet that it had received during the initial control period, the phospholipid content of the blood was 375 mg. A 10 day fast was then instituted and the phospholipid level was 328 mg. Essentially the same changes were observed in Dog N-ST. A 25 day fast led to but little change in the phospholipid content of the blood, and the observations made during a subsequent feeding period also failed to show any significant deviations in the level of blood phospholipids.

*Fatty Acids*—The maintenance of the level of total fatty acids with little change during fasting is clearly brought out, however, by Dogs N-PB and N-ST. In these the fatty acid values obtained during a control and fasting period may be compared with those obtained during subsequent feeding and fasting periods. The initial control values in Dog N-PB were 369 and 376 mg.; the values obtained during the first fast, which lasted for 30 days, fluctuated between 303 and 336 mg.; at the end of the 26 day feeding period this was 331 mg., and after the subsequent fasting period 330 mg. A similar tendency to resist change in total fatty acids is shown by Dog N-ST. The initial control values

were 349 and 359 mg.; seven values obtained during a 25 day fast fluctuated between 319 and 388 mg.; three values obtained during a subsequent period of 26 days in which the control diet was again administered were between 372 and 395 mg.

#### DISCUSSION

The results obtained in the present investigation clearly establish that a *lipemia* does not occur in the dog during fasting or during chronic undernutrition produced by severe reduction in the calorie intake. Numerous observations on animals fasted for 4 to 30 days failed to show a significant rise either early or late in any of the lipid constituents measured; namely, cholesterol, phospholipids, and total fatty acids. Numerous observations made during a state of chronic undernutrition that extended over several months and resulted in loss of as much as 50 per cent in body weight also failed to show a significant *increase* either early or late in the concentration of total fatty acids, cholesterol, or phospholipids in the blood. These results therefore fail to confirm the claims that a *lipemia* occurs in fasting.

Acute fasting in which all dietary constituents with the exception of salts and vitamin concentrates were withdrawn failed to produce much change in the concentration of total fatty acids, cholesterol, or phospholipid in the blood. The ability of the organism to maintain the level of these lipid constituents so near to the control level while such nutritional conditions were permitted to last for as long as 30 days is indeed worthy of note.

Chronic undernutrition, produced by limiting the intake of lean meat and carbohydrates (but not vitamins and salts) and extending over periods of about 5 months, resulted in more striking effects upon the level of the blood lipids than acute fasting that lasted for about 30 days or less. With few exceptions the concentration of cholesterol, phospholipid, and fatty acids was lowered at the end of an extended period of undernutrition. In a few cases the reduction in total fatty acids was quite severe. The loss in weight during such periods of chronic undernutrition was far greater than that observed during acute fasting, and it is by no means unlikely that the greater loss in weight may explain the more pronounced effects of chronic undernutrition. In no cases did acute fasting for periods of 18 to 30 days produce losses of more

than 30 per cent in body weight, whereas in chronic undernutrition as carried out in the present investigation losses in body weight as great as 50 per cent were not uncommonly observed.

#### SUMMARY

1. The changes that occur in the blood levels of total cholesterol, total fatty acid, and phospholipids were investigated (1) during acute fasting in which all dietary constituents with the exception of salts and vitamins were withheld for periods of 4 to 30 days, and (2) during chronic undernutrition produced by severe reduction in the caloric intake for periods of 16 to 18 weeks.

2. No lipemia or rise in the levels of total cholesterol, total fatty acids, and phospholipids was observed during acute fasting or chronic undernutrition.

3. Striking changes in the levels of total cholesterol, phospholipids, and total fatty acids in the blood were not observed during acute fasting that lasted for as long as 30 days.

4. Undernutrition prolonged until severe loss in weight occurs leads to a fall in the levels of cholesterol, fatty acids, and phospholipids in the blood.

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# THE RATE OF TURNOVER OF PHOSPHOLIPIDS IN KIDNEY AND LIVER

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Within the past few years three new and quite distinct methods have been used for the study of the rate of turnover of the phospholipids in animal tissues. In one elaidic acid, the *trans* isomer of oleic acid is employed (1), in another deuteriumated fatty acids are employed (2), and in the third radioactive phosphorus is used (3-6). All three methods are based on the same principle. One of the building stones of the phospholipid molecule is labeled so as to make it distinguishable from those normally present in the body; this labeled constituent is fed and the rate at which it becomes incorporated into the phospholipids in an organ is taken as a measure of their rate of turnover. Strictly speaking elaidic acid and deuteriumated fatty acids can serve only as a means of measuring the rate of exchange of the fatty acids, while radioactive phosphorus, on the other hand, can serve only as a means of measuring the exchange of the phosphoric acid in the tissue phospholipids.

Now it is obvious that the exchange of the various constituents of the phospholipid molecule *need* not proceed at the same rate. When reduced to its simplest terms, the turnover of phospholipid in any given organ can be regarded as being due to one or both of two fundamentally different chemical processes. On the one hand, it is possible to conceive of the phospholipids as being in a state of dynamic equilibrium with each of the constituents of the molecule. The rate of exchange of each constituent would depend upon the rate of hydrolysis of each of the linkages in the phospholipid molecule. If these rates of hydrolysis should be markedly different, so too the rates of exchange would be different. On

the other hand, the phospholipids in the tissue cells may conceivably undergo an irreversible degradation; as by oxidation. Since the amount of phospholipid present is known to remain substantially constant, the degraded phospholipid must be continually replaced. This replacement might be due either to synthesis within each individual cell, or to diffusion of phospholipid from the blood stream. In either case the rate of appearance of new phospholipid would be proportional to the rate of degradation, and the rates of exchange of all the constituents of the phospholipid molecule would be the same.

In estimating the rates of turnover of the phospholipids or of any other tissue constituent by means of labeled molecules, it is obvious that the apparent rate of turnover will be a function, not only of the real rate, but also of the relative concentrations of labeled and non-labeled molecules. It is only when and if the relative concentrations remain constant over a reasonable period of time that the apparent and real rates of turnover are identical. Furthermore, a comparison of the rate of breakdown and replacement of the phospholipids in one organ with that in another, on the basis of the apparent rates of turnover, is valid only if the relative concentration of labeled and non-labeled molecules is the same in all cases.

The ultimate purpose of the study of the turnover of the phospholipids is to disclose their functions in the animal body. From what has been said, it is very evident that it would be of considerable importance to know the comparative rates of exchange of all the constituents of the tissue phospholipids.

Thus far the most extensive studies have been made with elaidic acid and with radioactive phosphorus. The results obtained by these two methods are, in some respects, in quite good agreement. Thus both show clearly that in the intestinal wall (3, 5-8), in the liver (1, 3, 5, 6), and in the blood plasma (9, 10), the phospholipids, taken as a whole, have a rapid turnover.

In the case of other organs, the rate of exchange of the phosphoric acid appears to be very definitely more rapid than that of the fatty acids. For instance, in the carcass of the rat the exchange of phosphoric acid is about two-thirds complete within 1 day (5). The uptake of elaidic acid in rat skeletal muscle, on the other hand, is only about one-half complete in 4 days (1). The most striking difference seemed to be in the kidney.

In the course of work done some years ago on the rate of turnover of the phospholipids of intestinal mucosa and blood of cats (7, 9), some analyses were also carried out on the livers, kidneys, and hearts. The data obtained are given in Table I. It is evident that in all three organs the iodine number of the solid fatty acids increased above the level found in the controls, even as early as the 8th hour after feeding elaidin. Elaidic acid had therefore entered into the phospholipids of these organs. However, except in the case of the liver, further uptake of elaidic acid on continued feeding of elaidin was rather slight. And even in the liver the maximum level of elaidic acid was not reached after 5

TABLE I

*Elaidic Acid in Phospholipid Fatty Acids in Various Organs of the Cat*

Absorption time	Liver			Kidney			Heart		
	Solid acids	I No.	Elaidic acid	Solid acids	I No.	Elaidic acid	Solid acids	I No.	Elaidic acid
	per cent		per cent	per cent		per cent	per cent		per cent
Control	41.5	10.6	4.9	36.3	9.7	3.9	26.9	8.1	2.4
"	41.7	9.0	4.2	35.0	11.9	4.6	28.1	9.2	2.9
"	41.9	11.8	5.5	36.8	9.2	3.8	29.9	12.3	4.1
"	38.4	8.8	3.8	38.5	8.9	3.8	26.0	15.3	4.4
"	41.4	11.0	5.1	36.2	8.1	3.3	27.7	9.4	2.9
8 hrs.	40.0	19.6	8.7	39.2	15.6	6.8	28.8	24.0	7.7
18 "	41.5	22.4	10.4	37.8	16.4	6.9	24.2	14.9	4.0
48 "	44.0	41.4	20.2	41.6	19.8	9.2	26.7	21.3	6.3
120 "	44.2	35.5	17.5	40.2	19.5	8.7	24.4	21.6	5.9
2 wks.	46.8	59.4	30.8						

days. In rats, on the other hand, it had been found that the maximum level of elaidic acid, indicating complete turnover, is reached in 1 or 2 days (1). Accordingly it was realized that the apparently slow turnover of phospholipids in the kidneys and heart of the cat may have been due, in part at least, to the failure to maintain a sufficiently high and sustained absorption of elaidic acid over the entire experimental period. Further work had been planned.

Meanwhile Artom and coworkers (3) and Perlman, Ruben, and Chaikoff (5) had presented evidence to show that the phospholipids in the kidneys of the rat have a high rate of turnover as measured by radioactive phosphorus. In view of the very ob-



vious importance which would be attached to an instance in which the rate of exchange of the phosphoric acid was quite definitely different from that of the fatty acids in the phospholipids, it was decided to determine the rate of exchange of the phospholipid fatty acids of rat kidney by means of elaidic acid. The results of that study have clearly shown that the fatty acids in kidney phospholipid undergo a turnover which is of a lower order than do those of the liver and intestinal mucosa. Furthermore the rate of turnover of the fatty acids seems to be much slower than that of the phosphoric acid in the kidney phospholipids.

#### EXPERIMENTAL

When elaidic acid is used as a means of measuring the rate of turnover of tissue phospholipids, it has seemed best that the intake of elaidic acid be continuously maintained at a sufficiently high level so that mobilization of previously stored fat would be inhibited and fat metabolism would be borne entirely by the inflowing food fat. Otherwise the mobilization and metabolism of non-labeled fatty acids will lower the uptake of labeled fatty acids, in this case elaidic acid. To this end, the rats used for the following experiments were fed melted elaidin at frequent intervals throughout the day and in addition were offered a diet containing a high percentage of elaidin (Diet 290 (11)). The amount of melted elaidin given was calculated to be about the maximum amount that would be absorbed. Actually some of the rats showed evidence of increased excretion of fat in the feces, indicating that the intake was probably in excess of the capacity of the small intestine to absorb elaidin.

Four separate experiments were carried out. Adult male rats of approximately the same weight and age were selected for each experiment. One group of rats weighed about 150 gm.; the other three groups weighed about 300 to 350 gm. Three of the groups were fed the melted elaidin dropwise. In spite of some slobbering of the fat, especially at first, this procedure was found to be preferable to the use of the stomach tube, which was used in one group. Many of the animals, even from the start, lapped down the melted fat as rapidly as it was administered. In the last two groups, about 1 to 1.5 gm. of elaidin was given to each rat every 4 hours over periods of 1, 2, 3, or 4 days.

At the end of each day, two rats were killed. The kidneys were pooled, weighed, ground with sand, and rinsed into a flask with 95 per cent ethyl alcohol. In some cases the pooled livers were also used. The total lipids were extracted and separated into acetone-soluble and acetone-insoluble fractions,  $\text{MgCl}_2$  being used to aid precipitation. The acetone-insoluble lipids were saponified in a centrifuge tube, with 0.2 cc. of saturated  $\text{KOH}$  dissolved in 6 cc. of 50 per cent ethyl alcohol. During saponification the alcohol evaporated down to about 2 cc. Water was added to bring the volume up to 3 cc. After  $\frac{1}{2}$  hour's saponification (carried out in a gentle stream of  $\text{N}_2$ ), 25 per cent  $\text{H}_2\text{SO}_4$  was added and the fatty acids were extracted with peroxide-free ethyl ether. The combined ether extracts were evaporated to dryness, under a stream of  $\text{N}_2$ , and the residue was finally dried over  $\text{H}_2\text{SO}_4$  in a vacuum desiccator. The solid residue was then extracted with hot acetone and centrifuged. The clear acetone solution of the fatty acids was evaporated to dryness under  $\text{N}_2$ ; the flask was cooled in the vacuum desiccator and then weighed.

To separate the fatty acids into solids and liquids, they were dissolved in 95 per cent ethyl alcohol in a 15 cc. centrifuge tube, boiled, and brought to a volume of about 6 cc., 0.6 mg. of lead acetate in alcohol for every mg. of fatty acid was added, and the alcohol was centrifuged hot to remove the slight precipitate which formed. The volume of alcohol was adjusted so that the fatty acid concentration was always 10 mg. per cc. The tube was then put away at  $15-16^\circ$  for at least 4 hours. The insoluble lead soaps were centrifuged out, redissolved in six-tenths of the original volume of alcohol, and again set away at  $15-16^\circ$ . The insoluble soaps were stirred up with 5 per cent  $\text{HCl}$  and the fatty acids were extracted thoroughly with ether. This ether solution was washed with water and transferred to a weighed, glass-stoppered 125 cc. flask. The ether was evaporated under  $\text{N}_2$ . The alcohol solution of the soluble lead soaps was evaporated under  $\text{N}_2$  until just barely dry. Then 5 per cent  $\text{HCl}$  and ether were added and the flask shaken repeatedly. The contents of the flask were transferred to a 15 cc. centrifuge tube and centrifuged. The clear or very slightly milky ether solution was aspirated into a stoppered centrifuge tube and shaken up with water. On centrifuging, the clear ether solution separated sharply. This

was aspirated into a weighed 125 cc. glass-stoppered flask and evaporated under  $N_2$ . After evaporation of the ether, the flasks containing the solid and liquid fatty acids were cooled in a vacuum desiccator and then weighed. The iodine numbers of the fatty acids were then determined immediately by the Rosenmund-Kuhnhehn method (12). The elaidic acid content was calculated from the iodine number and percentage of solid acids (1).

### *Results and Comments*

The data obtained from the study of the turnover of the phospholipids in rat kidneys are given in Table II. It will be observed (Column 4) that there is no indication of any change in the amount of phospholipid in the kidney as a result of the continuous ingestion and metabolism of large amounts of elaidin. On the other hand, the iodine numbers of the solid acids (Column 6) and the calculated percentage of elaidic acid in the phospholipid fatty acids (Column 8) clearly show that there is a progressive exchange of the fatty acids in the kidney phospholipids. However, if the percentages of elaidic acid after 3 days are compared with those found in rats which have been fed elaidin throughout their lifetime, it is equally clear that the rate of turnover is such that it reaches completion only after several days. By extrapolation of the rough curve which may be fitted to the data given in Table II, it may be estimated that the turnover in the kidney will be about 90 per cent complete at the end of a week.

The data in Column 5 (Table II) show that there is no clear cut change in the percentage of solid acids coincident with the incorporation of elaidic acid into the phospholipids of the kidney. It is true that the values seem to be somewhat higher in the rats fed elaidic acid than in the controls. This applies especially to those rats fed elaidin over a long period of time. Possibly a greater number of data would show that the differences are really significant. Nevertheless it is quite clear that most of the elaidic acid that is built into the phospholipids of the kidney takes the place of the fully saturated fatty acids. There is no evidence of any consistent change in the iodine numbers of the unsaturated fatty acids as the percentage of elaidic acid increases.

In Table III are presented some data showing the rate of increase in the elaidic acid content of the livers of rats. Those in

Group A were secured on a group of rats which were given melted elaidin by mouth every 6 hours over periods ranging from 12 to 26 hours. The rats in Group III are the same as those used for the study of the rate of turnover of kidney phospholipid.

TABLE II  
*Turnover of Kidney Phospholipid in Rats*

Group No.	Remarks	Time on elaidin	Content of phospho- lipid fatty acids	Per cent of solid acids	I No.		Elaidic acid
					Solid acids	Liquid acids	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		<i>days</i>	<i>per cent</i>				<i>per cent</i>
Controls	Purina Fox Chow diet		1.87	33.8	6.5		2.4
			1.95	35.7	7.4	218	3.0
I	Fed melted elaidin dropwise at 9 a.m.	1	2.00	33.7	19.7	202	7.4
	and 1, 5, and 9 p.m.	1	2.00	35.0	22.2	207	8.6
		2	1.98	39.3	35.6	220	15.5
		2	1.95	39.7	37.3	218	16.5
II	Slightly anesthetized with ether; fed 1.5-2.5 cc. melted elaidin at 8 hr. intervals	1	1.93	35.5	19.2	213	7.4
		2	1.91	36.3	29.0	211	11.5
		3	1.94	37.8	32.2	206	13.5
III	Fed 0.9-1.1 gm. melted elaidin dropwise every 4 hrs.	1	1.99	36.1	26.7	204	10.7
		2	1.96	37.4	33.3	210	13.8
		3	1.82	38.1	39.0		16.5
IV	Fed 1-1.5 gm. melted elaidin dropwise every 4 hrs.	1	1.91	36.5	25.1	223	10.2
		2	1.82	38.1	33.2	218	14.1
		3	1.87	39.5	35.7	221	15.7
		4	1.97	38.8	36.3	223	15.7
	Fed Diet 290-C, high in elaidin (11) from weaning age for at least 10 wks.		1.95	41.0	57.6	204	26.3
			1.87	42.0	57.9	211	27.0
			1.88	41.7	58.6	214	27.2
			1.87	38.4	53.0	199	22.6

On comparing the percentages of elaidic acid in the liver phospholipids of Groups A and III with those found in rats which had been raised on a high elaidin diet, one will see at once that as early as 18 hours after the first dose of elaidin, maximal values are reached. Unfortunately there is a rather large variation from one animal to another, even in those which had been on a

standard diet for many weeks.<sup>1</sup> In spite of this variation, the results in Table III bear out the earlier observation (1) that the turnover of liver phospholipid, as measured by the elaidic acid method, is substantially complete within 1 day. Certainly the contrast in the rates of turnover of liver and kidney phospholipid is very striking.

TABLE III  
*Turnover of Liver Phospholipid in Rats*

Group	Remarks	Time on elai- din	Con- tent of phos- pho- lipid fatty acids	Per cent of solid acids	I No.		Elaidic acid
					Solid acids	Liquid acids	
		hrs.	per cent				per cent
Con- trols	Average of 8 analyses given in Table II (1)			37.0±1.9	6.9±1.3		2.8±0.5
A	Elaidin dropwise every 6 hrs.	12	2.84	37.8	28.2	244	11.9
		12	2.67	37.8	45.1		19.0
		18	2.57	34.8	46.3		17.9
		18	2.71	42.7	53.9		25.6
		18	2.83	41.3	51.7		23.7
		24	2.59	41.5	44.9		20.7
		26		40.3	38.8	228	17.4
		26		42.3	49.2		23.1
III	Elaidin every 4 hrs.	24	3.16	43.0	42.6	252	20.4
		48	2.85	39.8	46.0	251	20.4
		72	2.63	43.3	47.2	262	22.8
	Fed high elaidin Diet 290-C (11) from weaning until at least 13 wks. of age		2.74	43.9	56.0	225	27.3
			2.60	47.8	60.5	235	32.1
			2.64	43.9	52.9		25.8
			2.74	44.2	52.9		26.0
			2.98	41.4	51.5	240	23.7
			2.80	41.6	50.3	238	23.3

In the case of the liver there is a significant decrease in the percentage of liquid acids with the increase in the elaidic acid content. As there is no indication that a high percentage of solid acids coincides with an increased amount of phospholipid

<sup>1</sup> As duplicate analyses give reasonably consistent results, there seems to be no reason to suspect that the analytical procedure is responsible for the variation from one animal to another.

in the liver, the decrease in liquid acids must be due to partial replacement by elaidic acid. However, in the liver as in the kidney, most of the elaidic acid that is built into the phospholipids replaces fully saturated fatty acids. It can be calculated that when the elaidic acid has reached its apparent maximal percentage, the fully saturated fatty acids are reduced from 37 to 18 per cent and the unsaturated acids from 63 to 56 per cent, on the average. At the present time there does not seem to be any purpose in further speculation (1, 7) about the mechanism underlying the replacement of the natural fatty acids by elaidic acid. The study of the problem is being continued.

#### DISCUSSION

The results of this investigation have clearly shown that, as measured by the elaidic acid method, the turnover of fatty acids in the phospholipids in the liver may be substantially complete within 1 day, while in the kidney it is only about 60 per cent complete in 3 days. This rate of exchange of the fatty acids is in sharp contrast to the apparent rate of exchange of the phosphoric acid in the phospholipids of both organs (5). The peak in radioactive phosphorus content is reached in 5 to 10 hours in the case of the liver and in about 24 hours in the case of the kidney.

It is obvious that in making a comparison of the rates of exchange of the fatty acids and the phosphoric acid in the phospholipids it would be best to carry out the determinations on the same animals under exactly the same conditions. Indeed, the conditions in Perlman and coworker's experiments (5) are so different from those in the present study that it may be doubtful that the results should be compared. However, the apparent rate of turnover of the fatty acids is so distinctly different from that of the phosphoric acid that it seems very unlikely that the two really proceed at the same rate and are always mutually dependent on one another.

According to the hypothesis that the phospholipids act as intermediaries in fatty acid transport and catabolism, one must visualize a rapid turnover of the fatty acids in such metabolic phospholipids. For the purpose of testing that hypothesis, the direct methods of estimating fatty acid exchange, such as the elaidic acid method, would seem to be most suitable. In the

case of the intestinal mucosa, the liver, and the blood plasma the fatty acids in the phospholipids do undergo a rapid turnover. It has seemed best therefore to conclude that, in these organs, phospholipids do act as intermediaries in fatty acid metabolism. On the other hand, the rate of turnover of the fatty acids in the phospholipids of the muscles (1) and, as the present results show, of the kidneys as well is considerably slower than the assumed rate of fatty acid catabolism in these organs. If one is correct in assuming that fatty acids enter the muscles and kidneys, either free or combined, before combustion begins, and also that fatty acids serve as a predominant source of energy to these cells when the diet is rich in fat, then the slow turnover of phospholipid fatty acids in muscles and kidneys must be related, not to fatty acid catabolism, but to those rather indefinite reactions which are summed up under the term wear and tear. And if wear and tear are responsible for the fatty acid exchange in kidney phospholipid, then it is easy to imagine why the rate of exchange of the phosphoric acid in the phospholipids can be quite different.

At this time it perhaps should be pointed out that, although a rapid turnover of fatty acids in the phospholipids of an organ is certainly consistent with the hypothesis that phospholipids in that organ are acting as intermediaries in fat metabolism, it need not be regarded as proof that they do so. It is possible to imagine that the turnover of the fatty acids in the phospholipids of all the organs of the body is fundamentally due to the same process which goes on at different rates from one organ to another. Whether or not one wishes to attach a physiological function to the turnover of fatty acids or, as Weissberger has done (13), to that of phosphoric acid in the tissue phospholipids is mainly a matter of personal predilection.

#### SUMMARY

The rate of turnover of the fatty acids in the phospholipids of rat kidneys, as measured by the elaidic acid method, has been found to be comparatively slow. After 3 days of continuous elaidin ingestion, the uptake of elaidic acid by kidney phospholipids is only about 60 per cent complete. It may be estimated that the turnover would be about 90 per cent complete at the end of 1 week.

The rate of turnover of liver phospholipids, as in previous work, has been found to be quite rapid. The maximal uptake of elaidic acid by liver phospholipids occurs as early as 18 hours after its ingestion.

The interpretation of the rates of turnover of the various constituents of the phospholipid molecule is discussed.

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# THE RATE OF TURNOVER OF LECITHINS AND CEPHALINS IN THE LIVER\*

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The fact that the phospholipids in animal tissues consist of three distinct types of compounds, the lecithins, cephalins, and sphingomyelins, was established by the pioneer work of Thudichum (1). And yet, even to-day, except for the specific rôle that the cephalins play in the process of blood coagulation, nothing is known concerning the precise functions of each of these three types of phospholipids.

When it became evident that the rate of turnover of the phospholipids in such organs as the liver, intestinal mucosa, and blood plasma (2, 3) is sufficiently rapid to make it probable that in these organs phospholipids act as intermediaries in fatty acid transport and metabolism, whereas in other organs such as the muscles (2) the turnover is too slow, it was suggested that the phospholipids in the various organs of the body may be comprised of two functionally distinct types, the *metabolic* and *non-metabolic* phospholipids. The relative proportions of these two types were assumed to vary from one organ to another. Now, it was fully realized that if such a functional differentiation between phospholipids does hold, it must have a chemical basis. It was natural therefore to wonder whether it might be possible that the nature of the nitrogenous base, which is known to influence the physicochemical properties of the phospholipid molecule, also determines its rôle as an intermediary metabolite or as a structural component in the tissue cells. The chemical make-up of the sphingomyelins would seem to exclude them from consideration as metabolic phospholipids.

\* The results contained in this paper were presented before the American Society of Biological Chemists at Toronto, 1939.

From the beginning it had been found that the maximal percentage of elaidic acid in the phospholipids, except in the case of the intestinal mucosa, was about 35 per cent, even though the elaidin which was fed contained about 85 per cent of elaidic acid. It was thought therefore that the composition of the non-metabolic component of the liver phospholipids might be more or less fixed and that the whole of the elaidic acid would be found in the metabolic component. And since, for a number of reasons, it had been concluded that the lecithins, rather than the cephalins, were more likely to be metabolic in function, it was hoped that all of the elaidic acid in the liver phospholipids would be found in the lecithins. An experiment showed, however, that the elaidic acid content of the alcohol-soluble fraction of the phospholipids was almost the same as that of the alcohol-insoluble fraction. Nevertheless it was decided to continue with the investigation and especially to concentrate on the determination of the comparative rates of increase in elaidic acid in the lecithin and cephalin fractions of the liver phospholipids. Despite its shortcomings, it was necessary to depend upon the relative insolubility of cephalins and the solubility of lecithins in cold absolute alcohol as a means of fractionation.

#### EXPERIMENTAL

Rats weighing 200 to 300 gm. were fed melted elaidin dropwise every 6 hours for periods ranging from 12 to 48 hours. In addition they were offered a diet rich in elaidin (Diet 290 (4)). After the allotted time, the animals were killed and the livers were extracted to remove the lipids. The ether-soluble lipids were separated into acetone-soluble and acetone-insoluble fractions according to the standard procedure (2). The insoluble fraction was transferred to a 15 cc. centrifuge tube, reprecipitated with acetone, and then boiled with 5 cc. of absolute ethyl alcohol. The volume was adjusted to 3 cc. and the tube set away at about 4° overnight. After centrifuging, the precipitate was stirred up in 1 cc. of fresh absolute alcohol, cooled, and again centrifuged. The alcohol-insoluble (cephalin) fraction and the alcohol-soluble (lecithin) fraction were saponified, and the fatty acids extracted, purified, and separated into solid and liquid acids by the procedure already described (5).

## Results

The data showing the percentage of lecithin as calculated from the sum of the lecithin and cephalin fatty acids, the percentage and iodine number of the solid acids, and the percentage of elaidic acid are given in Table I. It may be seen that, by the procedure used, 51.3 to 66.0 per cent of the total phospholipid fatty acids was found in the lecithin fraction.

In the hope of getting some idea of the degree of fractionation of the liver phospholipids into lecithins and cephalins on the

TABLE I  
*Turnover of Lecithins and Cephalins in Liver of Rats*

Time on elaidin	Lecithin fatty acids				Cephalin fatty acids		
	Per cent of total	Solid acids		Per cent of elaidic acid	Solid acids		Per cent of elaidic acid
		Per cent	I No.		Per cent	I No.	
12 hrs.	58.2	38.3	30.2	12.9	37.1	25.5	10.5
12 "	56.7	39.4	47.2	20.7	35.8	42.3	16.8
18 "	64.8	34.9	48.1	18.6	34.5	43.0	16.5
18 "	58.0	44.6	56.0	27.8	40.0	50.6	22.5
18 "	57.1	42.9	54.1	25.8	39.3	48.2	21.1
24 "	62.6	45.6	50.6	25.6	39.1	40.9	17.8
26 "	53.5	41.3	42.4	19.5	39.2	34.4	15.0
26 "	51.3	43.8	52.9	25.7	40.8	45.0	20.4
48 "	66.0	47.7	56.5	30.0	41.0	45.1	20.6
8 wks.*	57.3	43.6	55.7	27.0	42.5	49.1	23.2
8 "	53.8	43.5	55.6	26.9	42.1	49.5	23.2

\* Raised from weaning age on Diet 290-C (4).

basis of the solubility in absolute alcohol, a few determinations were made of the choline content of the aqueous solution left after saponification and extraction of the fatty acids with ether.<sup>1</sup> The Beattie method of precipitating the choline as the reineckate and estimating it colorimetrically was used (6). In some cases the choline was estimated directly on an aliquot of the aqueous solution; the remainder was neutralized, and then saponified with Ba(OH)<sub>2</sub> according to the procedure described by Williams *et al.*

<sup>1</sup> The author acknowledges the assistance of Mr. R. J. Fassina in estimating the choline contents of the aqueous residues.

(7). No significant difference was found in the choline content before and after further saponification. An average of 80 per cent (70 to 84 per cent) of the total lipid choline was found in the aqueous residues from the lecithin fractions. Part of the 20 per cent in the cephalin fractions may well have come from sphingomyelins which had been dissolved in the ether solution of the total lipids.

In every one of the eleven experiments it was found that both the percentage and the iodine number of the solid fatty acids were higher in the lecithin fatty acids than in the cephalin fatty acids. Accordingly the elaidic acid content of the liver lecithins is consistently higher than that of the cephalins. The difference ranged from 2.4 to 9.4 per cent and averaged 4.9 per cent.

When allowance is made for the lower maximal content of elaidic acid in the cephalin fraction, it is evident that the rate of uptake of elaidic acid is just about the same in the lecithins and cephalins of the liver. It is true that, when the elaidic acid content of the lecithins and cephalins is calculated as a percentage of the maximum, in all but one of the nine experiments the turnover is greater for the lecithins than for the cephalins. Nevertheless, the fact remains that these data indicate quite clearly that the cephalins in the liver undergo a rapid turnover. If the rate of turnover is a proper clue to function, then it must be admitted that the cephalins as well as the lecithins are intermediates in fat metabolism.

In view of the fact that the choline determinations indicated that approximately 20 per cent of the total choline came out in the cephalin fractions, it might be thought that possibly the elaidic acid in the latter was in reality all contained in the contaminating lecithins. However, a calculation based on the assumption that 20 per cent of the cephalin fraction consisted of lecithins and *vice versa* showed that, in the last two rats in Table I, the liver lecithins contained 30 per cent of elaidic acid and the cephalins contained 15 per cent. Furthermore, it had been found that in every case, although most of the liver phospholipid was soluble in boiling absolute alcohol, there was some that was not. Thinking that perhaps this insoluble fraction might contain a higher proportion of true cephalins than that which separated out on

cooling, the two were analyzed separately. In two experiments, carried out on the extracts from the pooled livers of rats on a high elaidin diet, the fraction insoluble in hot alcohol, amounting to 19 and 25 per cent of the total cephalin fraction, contained 17.7 and 19.0 per cent of elaidic acid; the cephalins which separated out of the alcohol on cooling contained 17.9 and 21.0 per cent, respectively. Thus there was no evidence to indicate that the true cephalins take up no elaidic acid. However, the question needs to be investigated further.

#### DISCUSSION

The idea that the lecithins might have a metabolic function and the cephalins a structural or at least a non-metabolic function evidently has appealed to a number of workers within the last couple of years. In a recent review Bloor (8) has summarized the evidence that lends support to the idea.

The results of this present study, however, are not in harmony with the view that the lecithins alone, and not the cephalins, are intermediates in fat metabolism. It is clearly shown that, although the total uptake of elaidic acid by the lecithins is greater than by the cephalins, the rates of exchange of the fatty acids in the lecithins and cephalins of the liver are practically the same.

#### SUMMARY

The rates of turnover of the fatty acids, as measured by the rate of uptake of elaidic acid, are practically the same in the lecithins and cephalins of the liver of the rat. On the other hand, the maximum percentage of elaidic acid is significantly greater in the lecithins than in the cephalins.

If the rate of turnover of the fatty acids is taken as a criterion of the function of the phospholipids, these results do not support the idea that the lecithins alone are intermediates in fat metabolism and that the cephalins have exclusively a non-metabolic function.

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# THE EXCLUSION OF ELAIDIC ACID FROM THE PHOSPHOLIPIDS OF THE TESTES AND ITS UPTAKE BY OTHER ORGANS OF THE RAT

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The use of elaidic acid as a means of studying the turnover of phospholipids in the animal body is based on the premise that, as it is readily absorbed and burned, elaidic acid is probably transported and otherwise treated in the body in a manner comparable to stearic and oleic acids, its closest chemical relatives. The original observation that elaidic acid rapidly becomes incorporated into the phospholipids of the liver and more slowly into those of the skeletal muscles and, after more prolonged feeding, makes up about one-third of the total phospholipid fatty acids of these organs (1) is believed to prove the correctness of that premise. A single exploratory experiment having demonstrated clearly that elaidic acid enters into the phospholipids of the kidneys, the heart, the blood plasma, and the red blood cells, it was thought that the entrance of elaidic acid into the phospholipids was common to all organs of the body, the rate of entrance being a function of, and therefore a measure of, the rate of turnover.

Some time later McConnell and Sinclair (2) found that the brain was an exception to all of the other organs studied. Even in rats which had been fed elaidin throughout their entire lifetime, including the intrauterine period, elaidic acid amounted to only about 10 per cent of the fatty acids in the lecithins and cephalins of the brain. Accordingly, it was concluded that, while there was probably a considerable selection exercised in the building up of certain phospholipids in all organs of the body, this process of selection was much more rigorous in the case of the brain. At the time it was felt that this high degree of selection in the brain was probably peculiar to nervous tissue.



Recently it has been discovered that in the testes of rats there is a still more rigorous selection in the building up of the cellular phospholipids. Elaidic acid is completely excluded. Apart from the fact that the absence of elaidic acid from the phospholipids of the testes would seem to indicate the absence of phospholipids acting as intermediates in fat metabolism, nothing is known as to the physiological significance or the mechanism of the selective synthesis of the phospholipids.

#### EXPERIMENTAL

The observation that elaidic acid did not appear in the phospholipids of the testes was first made on a group of rats which had been fed elaidic acid over a period of 1 to 4 days for the primary purpose of determining the rate of turnover of kidney phospholipid (3). In order to find out whether the non-appearance of elaidic acid was due to a very slow turnover or to a process of exclusion comparable to that in the brain, the testes of three groups of rats which had been raised on a high elaidin diet from 40 to about 280 gm. were analyzed. The procedure employed has already been described (3). The results are given in Table I. In all cases the testes of two or more rats were pooled.

The iodine numbers of the solid acids (Column 5) clearly show that there is no significant increase above the control level in the animals fed elaidin. The values, it is true, tend to be somewhat higher than the four controls, but the difference is so small that it would require a large number of experiments to test its reality. The percentages of solid acids (Column 4) also are practically the same in the controls and in those rats fed elaidin. Accordingly, since the percentage of elaidic acid is calculated from the percentage and iodine number of the solid acids, the values for elaidic acid (Column 7) are substantially the same in those animals fed elaidin as in the controls.

It had been observed that the testes of the first two groups raised on the high elaidin diet were small and flabby as compared with those of normal rats (Column 2). This could readily be attributed to the inadequacy of vitamin E. The percentage of phospholipid fatty acids (Column 3), on the other hand, appeared to be normal. However, to exclude the possibility that the absence of elaidic acid from the testicular phospholipids was due

to atrophy of the testes, the third group of rats was given  $\alpha$ -tocopherol.<sup>1</sup> The testes were quite normal in size and appearance. The phospholipid content was unchanged. The iodine number and percentage of solid acids in the phospholipid fatty acids were actually closer to the control values than in the other two groups.

When it became certain that elaidic acid was excluded in the building up of testicular phospholipid, it became clear that other

TABLE I  
*Absence of Elaidic Acid from Phospholipids in Rat Testes*

Dietary conditions (1)	Weight of testes per 100 gm. body weight (2)	Phospholipid fatty acids (3)	Per cent of solid acids (4)	I No.		Apparent elaidic acid (7)
				Solid acids (5)	Liquid acids (6)	
	gm.	per cent				per cent
Stock diet, Purina Fox Chow		0.830	35.0	8.7	210	3.4
		0.984	34.7	9.7	200	3.7
	0.88	0.986	36.0	9.7	219	3.9
		0.760	36.0	8.7	224	3.5
High elaidin diet, 1 day*	1.17	0.830	34.5	7.4	202	2.8
	1.00	0.820	33.8	8.8	223	3.3
Same, 2 days*	0.87	1.023	34.6	12.6	191	4.8
	0.63	0.819	33.2	12.9	210	4.8
" 3 " *	0.79	0.902	32.9	11.8	171	4.3
" 4 " *	1.08	0.790	34.5	10.1	225	3.9
Raised on high elaidin Diet 290-C (4)	0.56	0.945	31.8	11.7	194	4.1
	0.75		31.4	10.7	211	3.7
	1.12†	0.985	35.4	9.8	226	3.9

\* Fed elaidin every 4 hours in addition to the high elaidin diet.

†  $\alpha$ -Tocopherol added to diet.

organs of the rat should be studied. However, the necessity of having not less than about 30 mg. of phospholipid fatty acids for the elaidic acid determination makes such a study difficult even though the organs from several rats are pooled. To confirm a single determination made several years ago, hearts were analyzed. An analysis has been carried out on lungs. A summary of the data at hand concerning the percentage of elaidic acid in

<sup>1</sup> To every 100 gm. of Diet 290-C (4) were added 100 mg. of ephynal kindly donated by Hoffmann-La Roche, Ltd., Montreal.

the phospholipid fatty acids of the various organs of rats which had been raised on the high elaidin diet, No. 290-C (4), is given in Table II. It needs to be emphasized that the phospholipids of control animals fed on either stock diets or on synthetic diets containing common fats and oils have an apparent elaidic acid content of about 4 per cent.

On inspection of the values in Column 7 of Table II, it will be seen that the elaidic acid contents of the phospholipids of the small intestine, the liver, skeletal muscle, kidneys, heart, and red blood cells are almost the same. The low value for the blood

TABLE II  
*Comparative Uptake of Elaidic Acid by Various Organs of the Rat*

Organ (1)	No. of determina- tions (2)	Phospho- lipid fatty acids (3)	Per cent of solid acids (4)	I No.		Elaidic acid (7)
				Solid acids (5)	Liquid acids (6)	
		<i>per cent</i>				<i>per cent</i>
Small intestine	4	0.731	47.8	54.6	168	29.2
Liver	6	2.75	43.8	54.0	235	26.4
Skeletal muscle	4	0.691	40.6	61.0	210	27.5
Kidney	4	1.89	40.8	56.8	207	25.8
Heart	2	1.11	41.6	60.0	266	27.8
		1.70	37.2	58.8	272	24.3
Blood plasma	1		32.1	36.0	121	12.9
" cells	1	0.272	48.1	54.3	200	29.0
Lungs	1	1.46	47.8	36.3	185	19.3
Brain	1	3.16	30.9	20.3	189	7.0
Testes	3	0.965	32.9	10.7	210	3.9

plasma of the rat is questionable, since it is a single analysis and especially in view of the much higher values which are found in the blood plasma of cats (5). The low value for the lungs may be open to question, since it is a single determination; however, there is no reason to suspect its accuracy. The single determination on the brain was carried out recently and is in complete agreement with the extensive analyses published previously (2). Incidentally, the lecithins and cephalins of the brain (the ether-insoluble lipids having been removed) were analyzed separately. The cephalin fatty acids amounted to 60.6 per cent of the total phospholipid fatty acids. For the lecithin and cephalin fractions,

respectively, the percentages of solid acids were 32.4 and 29.8 per cent; the iodine numbers of the solid acids were 21.1 and 19.8; and the elaidic acid percentages were 7.6 and 6.6.

#### DISCUSSION

The clear cut evidence that elaidic acid is excluded from the phospholipids of the testes even more rigorously than from those of the brain raises anew the question of the physiological function of the non-metabolic phospholipids, the relationship between function and composition, and, above all, the mechanism by means of which the composition is controlled. It would appear certain that, since elaidic acid is the predominant fatty acid being burned in the body, its complete absence from the testicular phospholipids indicates that the latter must have exclusively a non-metabolic function. But as to what that function is, the present results offer no clue. Naturally, elaidic acid cannot be used to measure the rate of turnover of testicular phospholipids. According to Artom *et al.* (6) the turnover of the phosphoric acid, at any rate, is comparatively slow.

At the present time it is impossible to formulate any satisfactory hypothesis as to the probable mechanism by means of which elaidic acid is excluded from the phospholipids of the testes and yet is incorporated into those of most of the other organs of the body. The various possible explanations fall into two distinct groups. In one group are those which involve a selection of certain fatty acids from the mixture circulating in the blood stream. This selection might conceivably result from differential permeability of membranes or from enzyme specificity. Opposed to these is the assumption that the cells of the testes are quite impermeable to all fatty acids of whatever nature and in whatever compound. Those fatty acids which are required for phospholipid synthesis during growth would necessarily have to be synthesized from carbohydrate *in situ*.

#### SUMMARY

Elaidic acid is not incorporated into the phospholipids in the testes of rats, even though elaidin is fed during the entire period of growth from weaning age on. The exclusion of elaidic acid is more rigorous than had previously been observed in the brain.

In all other organs studied—small intestine, liver, skeletal muscle, kidneys, plasma and red cells of the blood, and lungs—elaidic acid becomes incorporated in large amounts into the phospholipids.

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# GLYCINE AND HISTIDINE FEEDING AND CREATINE, CREATININE, AND INORGANIC PHOSPHORUS EXCRETION IN MAN

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The relation of glycine to creatine metabolism is of particular interest because of the fact that the ingestion of glycine by persons with progressive muscular dystrophy results in an increased excretion of creatine, and, sometimes, in a feeling of increased muscle strength. Some investigators (1-3) have observed an increase in the urinary creatine or total creatinine of normal adult men following the ingestion of glycine, while others (4-7) have not observed this effect.

Data reported upon the urinary creatine-creatinine following the administration of histidine are also of a contradictory nature (1, 8-15). In most of these studies animals, rather than human subjects, have been used.

This is a report of two types of experiments to determine the effect of the ingestion of glycine upon the creatine and creatinine excretion of normal men and women, and of one experiment to determine the effect of the ingestion of histidine upon these urinary components in the case of a normal woman. Because of their low creatine tolerance women are especially suitable as subjects for studies of this type. In three of these experiments the urinary inorganic phosphorus was also followed.

## EXPERIMENTAL

In each of the experiments there were three periods: a preliminary period which served to show the normal excretion of creatine and creatinine by each subject, an ingestion period, and an after period. For a few days before and throughout the

duration of the experiments, the subjects ate well balanced, meat-free diets of constant composition containing milk, eggs, cheese, fruits, vegetables, and whole wheat bread. Complete 24 hour collections of urine were made and each collection was diluted to a fixed volume, except in a few cases when the collections exceeded this volume. Total and preformed creatinine were determined daily by the method of Folin (16). Purified creatinine zinc chloride was used as the standard. The total urinary nitrogen for each period was determined by the Kjeldahl-Gunning method. In the long continued ingestion experiments the urinary inorganic phosphorus was determined daily by the method of Fiske and Subbarow (17). From time to time qualitative tests were made for certain pathological constituents in the urine. These were negative, except as indicated in Tables I and III.

The glycine used was obtained from the Amino Acid Manufactures of the University of California at Los Angeles. The *l*-histidine monohydrochloride monohydrate used was obtained from the University of Illinois, Urbana. Both of these substances were of known purity. These supplements were administered with milk or with water at breakfast or at luncheon when given once daily, and at each of these meals when given twice daily.

The results of the experiments are summarized in Tables I to III. The data represent the average daily output of each component for the periods indicated except when the period consists of 1 day only. In that case the single day's output is given. The days of each period and the periods followed each other consecutively, except during the menstrual periods, when no collections were made, and except in the case of an occasional loss. Quantities of urinary creatine are always expressed in terms of creatinine.

#### DISCUSSION

*Prolonged Ingestion of Glycine (Table I)*—During a period of 53 consecutive days Subject 1 ingested an amount of glycine equivalent to 197 gm. of creatine. There was no significant change in the urinary creatinine throughout the experiment. In the period when 9.16 gm. of glycine (equivalent to 16 gm. of creatine) were ingested daily there was a slight increase in the creatine output, but the increase did not continue throughout the period. Begin-

TABLE I

*Effect of Prolonged Ingestion of Glycine upon Urinary Creatinine, Creatine, and Inorganic Phosphorus\**

Subjects 1 and 2 received meat-free diets of constant composition containing 13.61 gm. of nitrogen and supplying 2726 calories.

Subject No.	Days	Urine				Glycine ingested daily
		Total N	Inorganic P	Creatinine	Creatine as creatinine	
		gm.	gm.	gm.	gm.	gm.
1. Young woman, 65.7 kilos at beginning, 67.5 kilos at end of experiment	1-7	9.4	1.23	1.15	0.06	0.00
	8-14	9.9	1.15	1.23	0.08	0.00
	15-21	10.2	1.25	1.23	0.09	0.57
	22-28	10.0	1.27	1.21	0.07	0.57
	29-35	10.3	1.32	1.22	0.08	0.57
	36-42	10.3	1.27	1.27	0.10	1.15†
	43-49	10.2	1.25	1.24	0.09	1.15†
	50-56	9.5	1.26	1.20	0.10	2.29†
	57-63	10.2	1.30	1.23	0.09	4.58†
	64-67‡	10.7	1.21	1.24	0.12	9.16†
	68-74‡	9.5	1.29	1.27	0.10	0.00
	75-81	9.7	1.26	1.25	0.11	0.00
	82-90	9.5	1.29	1.21	0.10	0.00
2. Young woman, 57.8 kilos at beginning, 60.5 kilos at end of experiment	1-7	12.0	1.12	1.07	0.07	0.00
	8-14	12.2	1.14	1.09	0.07	0.00
	15-21	12.4	1.13	1.08	0.09	0.00
	22-28	12.8	1.11	1.12	0.08	0.57
	29-35	12.7	1.09	1.12	0.08	0.57
	36-42	12.9	1.16	1.12	0.09	0.57
	43-49	12.3	1.11	1.10	0.09	0.57
	50-56	12.9	1.15	1.12	0.10	0.57
	57-63	12.9	1.12	1.13	0.10	1.15†
	64-70	12.4	1.15	1.11	0.10	2.29†
	71-77	13.0	1.16	1.11	0.14	4.58†
	78-79	13.4	1.19	1.13	0.19	4.58†
	80-86§	11.9	1.17	1.09	0.15	0.00
	87-93	11.7	1.17	1.12	0.12	0.00

\* The figures represent the daily average output for the periods indicated.

† Divided into two equal doses.

‡ Benedict's test for urinary sugar gave a slight green precipitate at times during these periods.

§ Beginning with the latter part of this period 35 gm. of bread were omitted from the diet.



ning with the 1st day of this period the amounts of creatine (expressed as creatinine) excreted daily were 0.13, 0.13, 0.15, and 0.09 gm. During the first after period the daily excretion of creatine was like that of the early days of the experiment. Because of the normal daily variation in the creatinuria of women the above increases do not seem significant. In fact, on the 12th day of the after period 0.15 gm. of creatine (expressed as creatinine) were excreted by this subject, and on the 22nd day 0.13 gm.

During a period of 58 consecutive days Subject 2 ingested an amount of glycine equivalent to 149 gm. of creatine. In this case, also, there was no significant change in the urinary creatinine throughout the experiment. The urinary creatine increased somewhat when 4.58 gm. of glycine were ingested daily. Beginning with the 1st day of this period the amounts of creatine (expressed as creatinine) excreted daily were 0.10, 0.12, 0.13, 0.13, 0.15, 0.17, 0.16, 0.19, and 0.19 gm. The increase in creatinuria persisted at a gradually decreasing rate during the first after period, the amounts (expressed as creatinine) then excreted daily being 0.18, 0.17, 0.14, 0.15, 0.16, 0.12, and 0.14 gm. However, if creatine is one of the important products of the catabolism of exogenous glycine, a larger amount of urinary creatine would be expected during the ingestion of an amount of glycine equivalent to 72 gm. of creatine, and particularly since an amount of glycine equivalent to 77 gm. of creatine had been ingested during the 49 days preceding. The total amount of creatine (expressed as creatine) excreted by this female during the last 9 days of the ingestion of glycine and during the after period of 14 days exceeded the total amount of creatine excreted during the preceding 23 days by about 1.10 gm. Her creatine tolerance, determined by the method of Milhorat and Wolff (18), was 56 per cent. Assuming her tolerance for creatine, if synthesized from the ingested glycine, to be the same as for ingested creatine, the excretion of 1.10 gm. of extra creatine represents the metabolism of 2.5 gm. of extra creatine. And if this extra creatine had been synthesized from the ingested glycine by the Sasaki reaction (19), or by any reaction in which 1 molecule of glycine took part in the formation of a molecule of creatine, only 1.43 gm., or about 3.47 per cent of the amount of glycine ingested during this time, would have been needed for the synthesis.

During an unusual storage of creatine in the muscles one would expect an increased utilization of phosphorus in the formation of creatine phosphate. Brown and Imbrie (20) observed that

TABLE II

*Effect of Ingestion of Single Doses of Glycine upon Urinary Creatinine and Creatine\**

Subject No.	Days in period	Urine			Glycine ingested
		Total N	Creatinine	Creatine as creatinine	
		gm.	gm.	gm.	gm.
2. Young woman, 55.7 kilos	3	10.0	1.09	0.16	0.00
	1	10.3	1.13	0.16	9.16
	3	10.5	1.09	0.16	0.00
3. Young male adult, 67.6 kilos	8	10.0	1.71	0.02	0.00
	1	12.8	1.82	0.02	9.16
	2	11.3	1.70	0.04	0.00
	7	11.5	1.73	0.00	0.00
	1	12.0	1.65	0.01	9.16
	2	11.4	1.73	0.01	0.00
	9	12.3	1.32	0.02	0.00
4. Young woman, 62.2 kilos	1	14.0	1.32	0.01	9.16
	2†	13.3	1.36	0.03	0.00
	3	10.3	1.13	0.07	0.00
5. Young woman, 54.0 kilos	1	12.4	1.14	0.07	9.16
	3	10.5	1.14	0.04	0.00
	10	8.2	0.94	0.05	0.00
6. Middle-aged woman, 55.0 kilos	1	10.3	0.91	0.07	9.16
	3	8.6	0.92	0.03	0.00
	3‡	9.7	0.93	0.06	0.00
	1	9.5	0.96	0.06	9.16
	2	9.5	0.95	0.05	0.00

\* The figures represent the daily average output for the periods indicated.

† On the 2nd day of this period the energy value of the diet was increased by 482 calories and the nitrogen intake by 2.0 gm.

‡ Beginning with this period the energy value of the diet was increased by 372 calories and the nitrogen intake by about 2.0 gm.

creatine administration induced a fall in the output of urinary phosphate in proportion to the extent of creatine retention in the case of anesthetized cats. Stacey (21), after oral administration of creatine to human subjects, noted a definite fall in the inorganic

phosphate concentration of the blood plasma concomitant with a rise in its concentration of total creatinine. The data obtained in this study do not indicate any increase in the retention of phosphorus during the ingestion of glycine, and therefore do not

TABLE III

*Effect of Prolonged Ingestion of l-Histidine upon Urinary Creatine, Creatinine, and Inorganic Phosphorus\**

Subject 2 (a young woman weighing 55.7 kilos) received a meat-free diet of constant composition containing 11.9 gm. of nitrogen and supplying 2355 calories.

Days	Urine				l-Histidine† ingested daily
	Total N	Inorganic P	Creatinine	Creatine as creatinine	
	gm.	gm.	gm.	gm.	gm.
1-7	8.2	0.84	1.08	0.11	0.00
8-14‡	8.9	0.85	1.10	0.12	0.00
15-21	8.7	0.92	1.09	0.10	0.00
22-28	9.5	0.98	1.11	0.11	1.60
29-35	8.9	0.89	1.12	0.13	1.60
36-42	9.8	0.92	1.13	0.10	1.60
43-49	9.8	0.93	1.15	0.12	1.60
50-52	9.3	0.93	1.12	0.12	1.60
53-55§	8.9	0.94	1.11	0.12	3.20
56-62§	9.1	1.00	1.10	0.11	0.00
63-69	9.2	1.03	1.09	0.10	0.00
70-74	9.1	0.98	1.08	0.12	0.00

\* The figures represent the daily average output for the periods indicated.

† l-Histidine monohydrochloride monohydrate divided into two equal doses, and with each dose an amount of sodium bicarbonate to neutralize the hydrochloric acid completely.

‡ Beginning with the 3rd day of this period the energy value of the diet was increased by 170 calories and the nitrogen intake was increased by 3.45 gm.

§ Benedict's test for urinary sugar gave a slight green precipitate.

suggest an increased storage of creatine in the case of either of these two women.

*Ingestion of Single Doses of Glycine (Table II)*—The creatine tolerance of some of the subjects used in this phase of these studies was determined while they were receiving the constant, meat-free

diets. The creatine tolerance of Subject 2, who served in both types of glycine feeding experiments, was 56 per cent. Subject 3 excreted 0.09 gm. of extra creatine while ingesting 1 gm. of creatine daily, and exhibited a slight creatinuria on a meat-free diet when not ingesting creatine. Subject 5 excreted 0.10 gm. of extra creatine on the 2nd day of the daily ingestion of 1 gm. of creatine. The tolerance of Subject 6 for ingested creatine, determined by the method of Milhorat and Wolff (18), was 80 per cent. Notwithstanding the inability of some of these individuals to retain 1 gm. of ingested creatine, in no case did the ingestion of an amount of glycine equivalent to 16 gm. of creatine produce any significant change in the excretion of creatine. Similar results, which are not reported, were obtained in the performance of this type of experiment with other subjects.

*Prolonged Ingestion of l-Histidine (Table III)*—During this study the diet of Subject 2 was similar in composition to that which she received during the prolonged ingestion of glycine but was lower in calorie and nitrogen value. An amount of *l*-histidine equivalent to 1 gm. of creatine was ingested daily for 31 consecutive days, and twice this amount for the next 3 days. During this time the urinary excretion of inorganic phosphorus, creatine, or creatinine was not significantly different from that of the fore and after periods. Therefore there was no demonstration of an influence of exogenous histidine upon the synthesis or excretion of creatine or creatinine.

*Regarding Positive Benedict's Tests*—No explanation can be offered for the slightly positive tests for sugar in the urine of Subjects 1 and 2 during a part of the experiment. This occurred in the case of another subject when he took 9.16 gm. of glycine in a single dose. These persons had had occasional urine sugar tests made previous to the experiment and none had indicated sugar. After the completion of the experiments tests for sugar in the urine of these subjects were negative.

#### SUMMARY

The daily administration of an amount of glycine equivalent to 1 gm. of creatine exerted no noticeable influence upon the urinary creatine, creatinine, or inorganic phosphorus of two healthy young women, although the ingestion was continued for 3 and 5 weeks

respectively. Nor did the excretion of these components change sufficiently to support the hypothesis that exogenous glycine is changed to creatine or creatinine by normal women, even though the amount of glycine administered was increased by doubling the daily dose for successive periods until finally, in the one case, an amount of glycine equivalent to 16 gm. of creatine was ingested daily for 4 days, and, in the other case, an amount equivalent to 8 gm. of creatine was ingested daily for 9 days.

Single doses of an amount of glycine equivalent to 16 gm. of creatine produced no significant effect upon the excretion of creatine or creatinine of several normal men and women.

The daily administration of an amount of *L*-histidine equivalent to 1 gm. of creatine for 31 consecutive days and of an amount equivalent to 2 gm. of creatine for the next 3 days exerted no noticeable influence upon the urinary creatine, creatinine, or inorganic phosphorus of a normal young woman.

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# CHEMICAL AND METABOLIC STUDIES ON PHENYLALANINE

## II. THE PHENYLALANINE CONTENT OF THE BLOOD AND SPINAL FLUID IN PHENYLPYRUVIC OLIGOPHRENIA\*

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Phenylpyruvic oligophrenia is an inborn error of metabolism characterized clinically by mental deficiency and chemically by the excretion of phenylpyruvic acid in the urine. It was shown previously that the feeding of a diet rich in protein or the ingestion of phenylalanine, phenylpyruvic acid, and of phenyllactic acid to patients afflicted with this syndrome resulted in an increased excretion of phenylpyruvic acid in the urine (1). The present paper contains observations on the phenylalanine and phenylpyruvic acid contents of the blood of normal and oligophrenic individuals, on the phenylalanine and phenylpyruvic acid content of the spinal fluid of these patients, and on the effects upon these values of feeding protein, phenylalanine, phenylpyruvic acid, phenyllactic acid, and tyrosine.

### EXPERIMENTAL

#### *Methods*

For the quantitative determination of phenylalanine the following procedure was used. The blood or spinal fluid proteins were precipitated with 5 volumes of 10 per cent trichloroacetic acid; the precipitate was removed by centrifugation and washed

\* Aided by a grant from Child Neurology Research (Friedsam Foundation).



twice with 1 volume of 5 per cent trichloroacetic acid. The combined filtrates and washings were diluted to volume and suitable aliquots were evaporated to dryness in a porcelain dish on the steam bath. The estimation of phenylalanine was carried out on the residue by means of the nitration method previously described (2, 3), by use of the Evelyn colorimeter with Filter 560 m $\mu$ .

It was shown (Table I) that the amount of color developed is proportional to the amount of phenylalanine used and that tyrosine, tyrosine and glycine, an excess of an amino acid mixture consisting of tyrosine, glycine, glutamic acid, arginine, and histi-

TABLE I

*Proportionality of Amount of Color Developed by Phenylalanine*

All reactions were carried out four or more times; average values are given.

Amount of phenylalanine	Optical density; $L = 2 - \log G$ ( $G$ = galvanometer readings)*					
	Alone†	1 mg. tyrosine	2 mg. tyrosine	2 mg. tyrosine + 10 mg. glycine	Amino acid mixture	36 mg. protein hydrolysates
mg.						
0.5	0.194	0.187	0.187	0.194	0.201	0.201
1.0	0.409	0.420	0.432	0.409	0.420	0.444
1.5	0.585	0.620	0.620	0.620	0.620	0.502
2.0	0.824	0.824	0.796	0.770	0.854	0.796

\* From the table in "Notes on operation of the Evelyn photoelectric colorimeter."

† These figures are in terms of optical density ( $L$ ) and when divided by the respective concentration ( $C$ ) of phenylalanine give a constant value ( $L/C = K$ ).

dine, or a protein hydrolysate does not interfere with the determination. The use of the color filter makes unnecessary the removal of tyrosine and histidine as described by Kapeller-Adler (2).

Moreover, various amounts of phenylalanine were added to samples of blood from normal individuals and the samples were treated as described above. The recovery of phenylalanine is shown in Table II, in which the results are expressed in net values after deduction of the blank.

Phenylpyruvic acid was determined colorimetrically by the green color which develops when ferric chloride is added to an

aqueous solution of the acid (4). The test was performed as follows: 2 cc. of a 1 per cent aqueous solution of  $\text{FeCl}_3$  were added to 20 cc. of the trichloroacetic acid blood or spinal fluid filtrates. As the green color is unstable, the reading was made in the Evelyn colorimeter (Filter 620  $\text{m}\mu$ ) at the point of maximum deflection of the galvanometer. The reaction was found to detect as little as 0.005 mg. per cc. of phenylpyruvic acid or about 0.04 mg. per cc. of blood under the experimental conditions here followed.

*Feeding Experiment*—Seven male and nine female patients were studied during the course of this investigation; their ages varied between 4 and 40 years, their intelligence quotients being between 5 and 50. Four normal subjects were included as controls. The

TABLE II

*Recovery of Phenylalanine Added to Human Blood (10 Cc.)*

All reactions were carried out four or more times; average values are given.

Phenylalanine	Recovery	
	Experiment 1	Experiment 2
mg.	mg.	mg.
0.5	0.45	0.45
1.0	1.0	1.0
2.0	2.0	2.0
5.0	5.0	4.5
10.0	9.7	9.9

phenylalanine content of the blood and spinal fluid was determined after ingestion by fasting (16 hours) subjects of (a) a protein-rich meal consisting of 250 gm. of meat, 50 gm. of cheese, 200 cc. of milk, and various vegetables, (b) *dl*-phenylalanine, (c) phenylpyruvic acid, (d) *dl*-phenyllactic acid, and (e) *l*-tyrosine. In each experiment phenylalanine and phenylpyruvic acid were determined at the fasting level and 2, 4, and 6 hours following the ingestion of the compound. For each estimation four replicate determinations were carried out on two different samples of blood drawn at the same time.

The following results were obtained. Under the conditions of analysis, the blood obtained from normal fasting individuals contained so little phenylalanine that it could not be determined with

even a fair degree of accuracy. In contrast, blood from sixteen fasting patients afflicted with phenylpyruvic oligophrenia contained from 15 to 41 mg. of phenylalanine per 100 cc. The spinal fluid of five fasting patients contained from 4 to 12 mg. of phenylalanine per 100 cc. Phenylpyruvic acid was not found in the blood or spinal fluid in any instance.

The ingestion of a high protein meal by eleven patients resulted in an appreciable increase of blood phenylalanine in nine experi-

TABLE III  
*Feeding of Phenylalanine and Related Compounds*

Substance fed	Patient	Blood phenylalanine, mg. per 100 cc.			
		0 hr.	2 hrs.	4 hrs.	6 hrs.
<i>dl</i> -Phenylalanine, 5 gm.	M. M.	18	25	24	23
	M. B.	26	28	34	24
	C. S.	31	38	42	35
	W. C.	41	53	43	52
	W. K.	40	48	45	51
Phenylpyruvic acid, 5 gm.	M. K.	34	34	42	36
	M. B.	23	29	29	28
	W. R.	25	29	38	21
	W. K.	17	28		27
<i>dl</i> -Phenyllactic acid, 5 and 10 gm.	M. K.	40	48	45	51
	W. R.	24	31	27	28
	M. B.	36	42	43	45
<i>l</i> -Tyrosine, 10 gm.	Average, 3 patients	19	19	21	20
Controls					
<i>dl</i> -Phenylalanine, 5 gm.	Average, 3 controls	0-1	2-3	2-3	0-1
Phenylpyruvic acid, 5 gm.	S. T.	0-1	0-1	2-3	0-1

ments, averaging 6 mg. per 100 cc. of blood. In one instance, the level rose from 27 to 45 mg. per 100 cc. No phenylpyruvic acid was detected in the blood in any of these experiments.

The feeding of 5 gm. of *dl*-phenylalanine to five oligophrenic individuals resulted in an appreciable rise in blood phenylalanine, with a maximum of 30 per cent of the fasting level (Table III).

5 gm. portions of phenylpyruvic acid were fed to four oligophrenics. A distinct rise in blood phenylalanine was found in

each instance (Table III) but no detectable quantity of phenylpyruvic acid appeared in the blood after either phenylpyruvic acid or phenylalanine was fed.

The data presented in Table III show that the ingestion of tyrosine had no apparent influence on the phenylalanine content of the blood. The ingestion of phenylalanine or of phenylpyruvic acid by normal individuals resulted in a slight increase in the blood phenylalanine (Table III).

The phenylalanine content of the spinal fluid was determined in two patients 6 hours after the ingestion of 5 gm. of the amino acid. 12 and 9 mg. per 100 cc. of fluid were found as compared with 5 and 3 mg. respectively in the same patients under fasting conditions. It should be pointed out, however, that the latter values were not obtained on the same day. No phenylpyruvic acid was found in the spinal fluid before or after the feeding of phenylalanine.

While this work was in progress, it was found that phenyllactic acid gives almost as much color in the nitration procedure as does phenylalanine. It was necessary, therefore, to rule out the possibility that phenyllactic acid and not phenylalanine was measured in the foregoing studies. This was accomplished by the following experiments. 15 mg. of phenylalanine were added to 30 cc. of normal serum, the proteins were precipitated with trichloroacetic acid, and the filtrate was extracted with ether in a continuous extractor for 8 hours. The residue from the ether extract gave no color with the nitration method, while all of the color remained in the aqueous layer. 6 mg. of phenyllactic acid, as the calcium salt, were added to 15 cc. of normal serum. The proteins were precipitated with trichloroacetic acid and the filtrate was extracted with ether for 4 hours. All the phenyllactic acid was found in the ether layer. Other experiments of a similar nature carried out on normal blood after the addition of phenylalanine, phenyllactic acid, or both, showed that, on extraction with ether, phenylalanine remains in the water layer while phenyllactic acid is completely extracted by ether.

With this ether extraction procedure, it was found that no phenyllactic acid is present in the blood of fasting patients affected with phenylpyruvic oligophrenia. Moreover, the ingestion of 5, 5, and 10 gm. of *dl*-phenyllactic acid by three patients resulted

in an increase of blood phenylalanine, as shown in Table III, while neither phenyllactic acid nor phenylpyruvic acid was found in these samples of blood. Finally, the trichloroacetic acid filtrates from one each of the phenylalanine and phenylpyruvic acid feeding experiments were extracted with ether for 3 hours and the colorimetric procedure was applied to both aqueous and ethereal solutions. The purple color developed only in the aqueous solution, thus confirming that in each instance phenyllactic acid was not responsible for the increased values of the colorimetric reading.

### Comment

The data presented confirm in general the results of Fölling, Closs, and Gammes (5) who were the first to observe an increase in phenylalanine in the blood of patients with phenylpyruvic oligophrenia. The method of estimation used by these investigators, however, was not quantitative, since it was based upon the ability of *Bacillus proteus* to convert phenylalanine into phenylpyruvic acid, which was then determined qualitatively by the ferric chloride reaction. The experiments here reported seem to exclude the presence of appreciable amounts of phenylpyruvic acid in the blood and spinal fluid and of phenyllactic acid in the blood of these patients. It appears, therefore, that the *essential biochemical characteristic of the disease consists in an inability of the subjects to dispose of phenylalanine at a normal rate* rather than in a failure to break down phenylpyruvic acid, as had been previously assumed (1, 6). The presence of phenylpyruvic acid in the urine may be considered, then, as an incidental phenomenon resulting from the deamination of a portion of the blood phenylalanine by the kidney tissue. The observation that ingestion of *d*-phenylalanine results in a significantly higher urinary output of phenylpyruvic acid than does an equivalent quantity of the *l* acid (1) may be explained by assuming that a portion of the natural amino acid was utilized for some physiological process which may not have involved deamination to phenylpyruvic acid (*cf.* (7)).

The formation of phenylpyruvic acid in the kidney represents an alternative path in the catabolism of phenylalanine. This route would be available to the organism when the normal pathway is blocked. Some evidence which suggests that the normal

route of phenylalanine catabolism may be through tyrosine and not via phenylpyruvic acid is as follows: (a) studies in alkaptonuria have shown that both phenylalanine and tyrosine cause an increased elimination of homogentisic acid (8); (b) when the liver is perfused with phenylalanine, tyrosine is found in the perfusion fluid (9); (c) in tyrosinosis, the ingestion of phenylalanine causes an increased urinary excretion of tyrosine and of *p*-hydroxyphenylpyruvic acid (10); (d) experiments with tissue slices indicate that phenylpyruvic acid fails to give acetoacetic acid under conditions in which phenylalanine and tyrosine yield this compound (11). Other evidence of the close metabolic relationship between tyrosine and phenylalanine is given by the studies on experimental alkaptonuria (12, 13) and by the finding of *l-p*-hydroxyphenyllactic acid in the urine of vitamin C-deficient premature infants following the ingestion of either phenylalanine or tyrosine (14). In this connection, the apparent failure of the feeding of 10 gm. of *l*-tyrosine to change the level of blood phenylalanine of the oligophrenic patients is of interest. The opinion that different metabolic pathways which may vary according to the requirements of the organism are available is in agreement with recent observations on the metabolism of sulfur compounds (*cf.* (15)).

The results of feeding phenylpyruvic acid and phenyllactic acid are worthy of especial interest. These compounds, while increasing the phenylpyruvic acid output in the urine (1), fail to induce a rise of the ketonic acid in the blood, but cause instead an appreciable increase of blood phenylalanine. This finding appears to indicate that the *patients are able to aminate the keto and hydroxy acids and that the phenylalanine so formed is eventually deaminated in the kidney to phenylpyruvic acid.* Within the past few years evidence which indicates that keto acids probably are transformed into the corresponding amino acids in the animal organism has been accumulating. Thus, certain keto acids are able to support the growth of animals maintained on a diet deficient in the corresponding essential amino acid (16). Likewise, the methyl derivatives of many essential amino acids can be used for purposes of growth in lieu of the corresponding amino acid. The mechanism of their conversion appears to consist in the oxidation of the N-methylamino acid to the corresponding

ketonic acid, followed by amination to the amino acid (17-20). Our data also indicate the occurrence of such amination and deamination processes in man. The possibility that the alteration of the anabolic pathway of phenylalanine indicated by this investigation may result in a diminished availability of the amino acid in the building up of proteins remains the subject for a later paper of this series.

#### SUMMARY

1. Quantitative determinations of phenylalanine and of phenylpyruvic acid in the blood of sixteen patients with phenylpyruvic oligophrenia showed a content of phenylalanine varying from 15 to 41 mg. per 100 cc. and the absence of phenylpyruvic acid. Neither compound could be estimated in appreciable amounts in the blood of normal individuals by the procedures described.

2. The blood phenylalanine showed a significant increase following ingestion of proteins, of phenylalanine, of phenylpyruvic acid, and of phenyllactic acid.

3. The ingestion of these substances did not result in the appearance of determinable quantities of phenylpyruvic acid or of phenyllactic acid in the blood.

4. Patients with phenylpyruvic oligophrenia have phenylalanine but no phenylpyruvic acid in the spinal fluid.

5. Ingestion of phenylalanine causes an increase in the amount of phenylalanine in the spinal fluids of these patients.

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# THE PREPARATION OF RENAL EXTRACTS CAPABLE OF REDUCING THE BLOOD PRESSURE OF ANIMALS WITH EXPERIMENTAL RENAL HYPERTENSION

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Recent experimental studies have indicated that many forms of hypertension as induced experimentally or as it occurs in man are due to a derangement of normal renal function. The evidence available at present indicates that the abnormal kidney may liberate some substance which plays a part in causing the hypertension. However, the amount of normal renal tissue present in the body also determines whether or not hypertension occurs (1, 3, 4). The normal tissue is thus able to offset the effects of the ischemic tissue. This effect of the normal tissue may be explained as exerting itself through some humoral agency, the absence of which in the diseased organ gives rise to hypertension. The correctness of this view is suggested by three lines of evidence which have been reported elsewhere (5): (1) the capacity of suitably prepared renal extracts to inhibit the pressor effects of renin; (2) the spontaneous reduction of the blood pressure of hypertensive rats during the late stages of pregnancy due conceivably to the development of the fetal kidney; (3) the capacity of renal extracts to lower the blood pressure of hypertensive rats and dogs.

The present report describes a relatively simple procedure which we have found suitable for preparing renal extracts capable of eliciting the results enumerated above. Since the active principle

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is effective when administered orally, it is unnecessary to effect a high degree of purification of the final extract. The problem resides in concentrating the effective agent present in a large mass of renal tissue to a sufficiently small volume.

### Methods

Fresh kidneys derived from various animals—pigs, beef, and dog—have been used. Since pig kidneys are most readily available and cheapest, most of our work has been carried out with these. Immediately after removal from the body, the kidneys are finely ground into 0.25 N hydrochloric acid, approximately 2 liters of the acid solution being used for each kilo of tissue. The mixture is stirred occasionally, allowed to stand for at least 12 hours, and pressed through cloth. The residual glands are extracted once more with aqueous acid and again pressed. The insoluble glandular residue is now discarded.

The combined acid extracts are carefully neutralized with 5 N sodium hydroxide to a pH of 3.9 to 4.1. The purpose of adjusting the acidity to this point is to reduce the amount of inert protein. The mixture is now filtered through cloth, giving a clear yellow solution. This filtrate is acidified to reduce the pH to about 2.0 to 3.0. About 1.5 volumes of a saturated solution of ammonium sulfate are now added to the filtrate and the mixture set aside in the ice chest overnight.

The precipitate formed by the ammonium sulfate is collected on a Buchner filter and thoroughly dried. Covering the precipitate with a little water or sodium chloride solution before it becomes impacted on the filter permits the removal of much of the ammonium sulfate trapped in the precipitate. Each pound of kidneys yields about 1.5 to 3 gm. of precipitate at this stage. This crude precipitate is satisfactory for the treatment of rats and may be administered by incorporation into the animal's food.

To concentrate the active principle, the precipitate obtained as described above is finely powdered and extracted four or five times with an equal volume of 50 per cent aqueous acetone. The mixture is filtered after each extraction and the combined filtrates reduced *in vacuo* at 35–40° until all the organic solvent is removed. A lipoidal mass is deposited during this procedure, which contains but slight activity, if the ammonium sulfate content of the crude precipitate is not excessive.

The aqueous solution obtained as just described is chilled and filtered to give a dark yellow or light brown clear solution. This solution which is slightly acid is neutralized with 0.1 N NaOH and shaken with decolorizing charcoal<sup>1</sup> (about 2 gm. of charcoal per 100 cc. of solution) to remove the coloring matter. The clear, almost colorless solution is now again precipitated with ammonium sulfate as in the original procedure.

We have found the above procedures convenient for preparation of the extract in the laboratory. Several modifications and alternative procedures have also been used and will be described briefly. To preserve better the active principle, the kidneys may be ground with an organic solvent—acetone, alcohol, or benzene. The active principle is not appreciably soluble in these liquids and hence must be subsequently extracted with a neutral or acid aqueous solution after the organic solvent is removed. For this reason we have omitted the use of the organic solvent except where the glands were to be preserved for a long time before extraction.

Instead of using ammonium sulfate as a precipitant we have also precipitated the active material by saturating the acidified filtrate with sodium chloride. This procedure is more convenient and less expensive than that involving the use of ammonium sulfate but is less satisfactory in so far as final yields are concerned.

An alternative method, which we have also utilized, combines and simplifies the several steps outlined above but does not uniformly give as good yields and involves the use of a large volume of acetone (or alcohol). In this procedure, the acid extract of the kidney prepared as already described and brought to pH 4.0 is treated with an equal volume of acetone (or alcohol), and the mixture is filtered through cloth. The clear filtrate is acidified to pH 2.5 and an equal volume of saturated ammonium sulfate is added. This mixture is thoroughly shaken and allowed to stand for several hours in the refrigerator. The precipitate which forms at the zone of contact of the two layers and which contains much of the active principle is now collected and thoroughly dried on a Buchner funnel. The yield of the active substance obtained by this simplified procedure is less than that derived by the first method described.

<sup>1</sup> Charcoal adsorbs only small amounts of the active principle from neutral aqueous solutions. However, it adsorbs it very efficiently from a 50 per cent aqueous solution of alcohol or acetone.

Since the active principle is insoluble in organic solvents, it is also possible to remove it from aqueous solution by the addition of at least 10 volumes of acetone. Although useful for extracting small batches of glands and avoiding the use of ammonium sulfate,

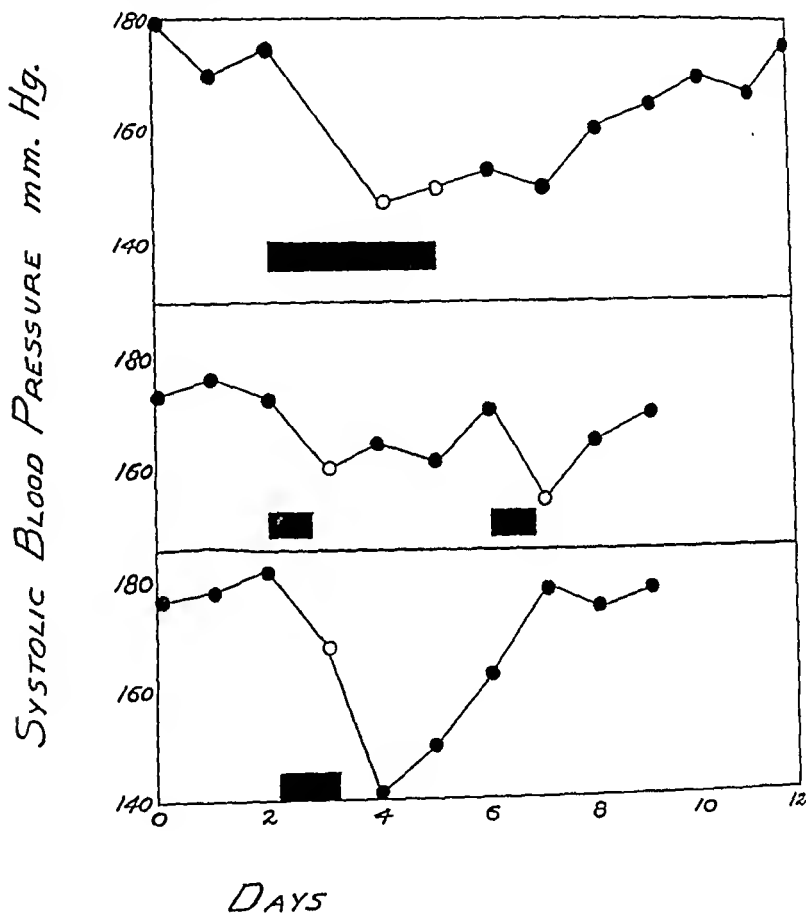


FIG. 1. The effect of renal extract administered orally on the blood pressure of hypertensive rats. The upper curve illustrates a moderate decline in blood pressure, sustained for several days, induced by the administration of renal extract. The middle curve represents a lesser decline of short duration induced on two separate occasions by feeding small amounts of an active fraction. The lower curve represents a marked reduction following the administration of a relatively larger amount of extract for a short period of time. The duration of administration is signified by the black blocks. Each curve represents average values obtained on a series of four rats. ● not receiving renal extract; ○ receiving renal extract.

this procedure is more laborious and expensive than that already described.

Only fresh glands have been found to yield active extracts. The active principle (as is also the case with many endocrine products) is apparently rapidly destroyed in the gland after removal from the body.

### *Results*

To determine the activity of the extracts obtained by the procedures described above, we have utilized the rat rendered hypertensive by partial nephrectomy according to the technique described by Chanutin and Ferris (2). Systolic blood pressures were determined by the procedure previously described (6).

In Fig. 1 is reproduced a typical experiment based on the average pressures obtained on three series of four rats each. As might be anticipated the reduction in blood pressure is dependent on its initial level. Thus a given amount of extract will lower the blood pressure of rats whose initial systolic pressures are 200, 180, and 160 mm., to 150, 140, and 130 mm. respectively. This reduction of the blood pressure by an amount which is approximately one-half the increment of pressure above 100 mm. serves as a convenient and sensitive method for determining the potency of a given extract. In general the extract obtained from a pound of kidneys suffices to lower the blood pressure of two to eight hypertensive rats to approximately normal levels.

There appears to be a seasonal variation in the content of the active principle similar to that observed in the case of thyroid, adrenal cortical, and other hormones. Application of the above methods to blood, liver, spleen, lung, and placenta yielded extracts of inappreciable potency.

Administration of the extract to dogs rendered hypertensive by the application of a Goldblatt clamp has likewise been followed by reduction in blood pressure. Several patients have also displayed a moderate decline in blood pressure following the ingestion of the extract. These results will be described in detail elsewhere.

### DISCUSSION

Unlike the non-specific depressor substances present in organ extracts, the substance with which we are concerned here does

not reduce the blood pressure of normal animals. The long period of its action and its activity when administered orally also sharply differentiate it from any of the known depressor substances. Further evidence, however, is necessary before it can be established that the principle present in our extracts is a normal physiological constituent of the kidney, the absence of which is responsible for the development of hypertension.

Only preliminary attempts have thus far been made to isolate the active principle and study its chemical properties. The active substance is relatively stable and, as evidenced by its effectiveness when administered orally, it is not of protein nature. It is relatively stable in aqueous dilute acid but loses its activity in alkaline solution.

The active principle with which we have been concerned differs from that utilized by several previous workers who prepared alcohol-soluble phenolic derivatives of renal tissue. These substances act only on parenteral injection and, we believe, through their toxic effects. They are discarded in our method of procedure.

#### SUMMARY

A method is described for the preparation of renal extracts which are capable of reducing the blood pressure of animals with experimental hypertension. The active principle is non-protein in nature, water-soluble, and is effective when administered orally. Its behavior is unlike that of any of the known non-specific depressor substances present in organ extracts. The latter are for the most part ineffective when administered orally and even such effects as are obtainable following parenteral injection are only of brief duration. Its solubility in water but insolubility in organic solvents also sharply differentiates the effective principle from many well known non-specific depressor substances present in tissue extracts.

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# THE VERATRINE ALKALOIDS

## VII. ON DECEVINIC ACID

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Until recently the study of degradation products from cevine has been confined to the mixtures obtained by pyrolytic procedures such as selenium dehydrogenation and soda lime distillation. However, in a recent (1) short communication the isolation was recorded of a non-nitrogenous acid,  $C_{14}H_{14}O_6$ , which resulted in appreciable yield by the oxidation of cevine with chromic acid. The study of this acid has now been carried further and, while confirming this formulation, has given information which affords a suggestion of its general nature. For convenience the designation *decevinic acid* has been adopted for this acid.

On being heated with sulfur at about  $300^\circ$ , decevinic acid was rapidly dehydrogenated and from the melt a crystalline substance was isolated (melting at  $245-246^\circ$ ) which appeared to be the major product and analysis of which agreed with a formula  $C_{12}H_6O_4$ . The formulation of the substance, its behavior towards alkali (2 moles were required), the phenolic color reactions exhibited by it, and its melting point suggested at once 2-hydroxy-1,8-naphthalic anhydride (1) which has already been described (2) with the melting point  $245-246^\circ$ . After saponification with stronger alkali it was degraded with loss of  $CO_2$  to a monobasic acid which corresponded in properties with those recorded for 2-hydroxy-8-naphthoic acid (2). The identity of the hydroxy-anhydride,  $C_{12}H_6O_4$ , was confirmed by methylation to a monomethyl ether,  $C_{13}H_8O_4$ , which was shown by comparison in properties and mixed melting points to be identical with 2-methoxy-naphthalic anhydride (2, 3). The latter was obtained by oxidation of 3-methoxyacenaphthenequinone (4). These observations,

barring rearrangements, at once indicate that decevinic acid must possess a hydronaphthalene ring system which in turn constitutes a portion of the cevine molecule. A study of the behavior of this acid towards alkali and also on acetylation and methylation has given information from which certain tentative conclusions may be drawn.

As previously reported (1), titration of decevinic acid in the cold with alkali has shown the presence of two acid groups or of an acid and a labile lactone group. When the acid was refluxed with excess 0.1 N alkali, no appreciable additional alkali was consumed. However, with N alkali an additional equivalent was required with degradation to the acid to be described below. With diazomethane decevinic acid yields, as reported, a dimethyl derivative. More recently with acetic anhydride an *acetyldecevinic acid* has been prepared. The latter on methylation gave an *acetyldecevinic methyl ester* which proved to be a monomethyl ester. Since the above dimethyl derivative could not be acetylated, the acetyl group must be assumed to occupy the same position as one of the methyl groups. The dimethyl derivative on careful titration in the cold consumed only 1 equivalent of alkali but when heated with 0.1 N alkali an extra equivalent was required. In the former case partial saponification occurred, since the product which was isolated proved to be a *monomethyl derivative* of decevinic acid. Like the original dimethyl derivative, this also could not be acetylated.

If the above acetyldecevinic methyl ester was refluxed with methyl alcohol, the acetyl group was removed with production of a *decevinic monomethyl ester* isomeric with the above monomethyl derivative. Contrary to the latter, its methyl group was so labile towards alkali that it behaved on direct titration like decevinic acid itself and consumed almost directly 2 equivalents of alkali. It appears, therefore, that acetylation must occur at the same point as that occupied by the more stable methyl group.

Two possible interpretations can be considered to explain these observations. Decevinic acid may be a dibasic acid in which one carboxyl group forms a more stable ester than the other. To explain the failure of the dimethyl or stable monomethyl derivative to acetylate, it would be necessary to assume that such acetylation can occur only on the carboxyl group which forms the

stable ester group; in other words, production of a mixed anhydride. Otherwise, decevinic acid must be a monobasic lactone acid containing a labile lactone group and also an enolic hydroxyl group which is the point of acetylation as well as of methylation. The latter alternative fits in best with a number of observations which have been made. The characteristic color reactions given with ferric chloride support the presence of an enolic hydroxyl group.

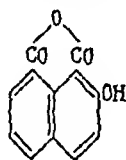
Of importance is the ready degradation of decevinic acid with alkali. Although under gentle conditions 2 equivalents of alkali are consumed, under more vigorous conditions a 3rd equivalent is used up and  $\text{CO}_2$  is split off with simultaneous addition of a mole of  $\text{H}_2\text{O}$  with the production of an acid,  $\text{C}_{13}\text{H}_{16}\text{O}_5$ , which no longer gives the prompt typical reaction with ferric chloride. This acid titrates as a dibasic acid (or monobasic lactone acid) and forms a *dimethyl derivative*. No other characteristic derivative was obtained from this acid. However, when distilled or heated with alkali, further degradation occurred with  $\text{CO}_2$  cleavage to a *keto-lactone*,  $\text{C}_{12}\text{H}_{16}\text{O}_3$ . This substance although neutral to sodium carbonate solution consumed 1 equivalent of alkali owing to cleavage of a lactone group. The presence of a carbonyl group was shown by the formation of both an *oxime* and a *phenylhydrazone*.

This series of degradations when considered with the production of a naphthalic anhydride derivative on dehydrogenation places limitations on the positions which can be assigned to the remaining carbon atoms other than the 1 and 8 carbon atoms of hydroxynaphthalic anhydride. Apparently, one of these must be an angular methyl group situated on a carbon atom common to both rings. The 2nd carbon atom could be attached either directly to the ring system as a carboxyl (a lactone) carbon atom or to a side chain from which one of the carboxyl groups of hydroxynaphthalic anhydride arises. In the latter case, this requirement would be met by an  $\alpha$ -keto acid (or lactone) side chain.

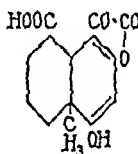
The behavior of decevinic acid towards alkali is perhaps more easily explained by the first assumption. In this case it would have to be a dibasic lactone acid. The fact that it requires more vigorous treatment with alkali for the consumption of a 3rd equivalent of alkali which involved loss of  $\text{CO}_2$  can be due either to

resistance to saponification or to rapid relactonization during titration under the gentler conditions. The production of the  $C_{13}H_{16}O_6$  derivative and in turn the ketolactone,  $C_{12}H_{16}O_3$ , can be simply explained by successive loss of carboxyl groups and final relactonization presumably on a double bond. There remain, however, certain observations which are difficult to harmonize with this interpretation. Among these is the fact that the condensation product with *o*-phenylenediamine described below must involve the lactone group and, if a glyoxaline derivative, would still be a dibasic acid, which is contrary to our experience.

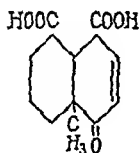
A second possibility is the formula (II) of an  $\alpha$ -ketolactone acid. This structure was first suggested by the fact that decevinic acid



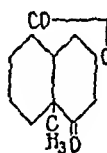
I



II



III



IV

was found to react readily with *o*-phenylenediamine to give in good yield a *condensation product*,  $C_{20}H_{20}O_5N_2$ , involving the loss of only 1 mole of water and which on titration behaved as a monobasic acid. If this product is a hydroxyquinoxaline derivative (or even a glyoxaline derivative) arising in a normal manner from the condensation with the  $\alpha$ -ketolactone group and simultaneous opening of the latter, only 1 mole of water would be lost. Such a condensation product would still be a monobasic acid. The conversion of such an  $\alpha$ -ketolactone acid on saponification with loss of  $CO_2$  and addition of water would require certain involved rearrangements in order to lead to a structure (III) for the dibasic acid  $C_{13}H_{16}O_6$ . The latter on decarboxylation and lactonization could then give a structure (IV) for the ketolactone,  $C_{12}H_{16}O_3$ .

Such interpretations can be at the moment only tentative and it is hoped to obtain other clarifying evidence.

Hydrogenation studies with this series of substances, although still incomplete, have given results which will have to be considered in the final interpretation of their nature. Decevinic acid on hydrogenation yielded a mixture which could not be directly crystallized. After low pressure distillation, however, accompanied apparently by water cleavage, a substance was obtained which after recrystallization gave analytical figures agreeing with a formulation  $C_{14}H_{20}O_4$ . Although on direct titration 2 equivalents of alkali were consumed, it gave only a *monomethyl ester*,  $C_{15}H_{22}O_4$ . The acid must therefore be a *monobasic lactone acid*.

The above ketolactone,  $C_{12}H_{16}O_3$ , on hydrogenation yielded a mixture from which two substances were isolated for which the provisional formulas  $C_{12}H_{18}O_2$  and  $C_{12}H_{18}O_3$  have been derived. The former was produced presumably by reductive cleavage of the lactone group to the desoxy acid and subsequent relactonization on the hydroxyl group produced by reduction of the carbonyl group. The substance  $C_{12}H_{18}O_3$  represents possibly an intermediate step.

The above observations are by no means sufficient to permit final conclusions as to the structure of decevinic acid. The possible origin of a hydronaphthalene derivative from the ring system of cevine will be considered elsewhere in the discussion of results obtained in the degradation of this alkaloid by other methods.

#### EXPERIMENTAL

*Dehydrogenation of Decevinic Acid*—A well ground mixture of 1 gm. of decevinic acid and 0.23 gm. of sulfur was placed in a test-tube with the upper end sealed to a smaller glass tube to act as an air condenser. Before heating, the air was replaced by nitrogen. The temperature of the salt bath was slowly raised until gases were evolved. This occurred suddenly at about  $300^{\circ}$  and was rapidly ended. After 5 minutes heating was interrupted. 200 cc. of gas were evolved. Nothing appeared to condense in the tube above the reaction mixture with the exception of a small amount of crystalline material just above the melt. This suggested absence of water formation during the dehydrogenation.

The melt was broken up and extracted with boiling acetone. This extract after evaporation was fractionated in a molecular still. 0.32 gm. of material distilled up to an oil bath temperature of 150° under a pressure of 0.001 mm. or less. Upon recrystallization from acetone 0.145 gm. of yellow needles was obtained which had a micromelting point of 245–246°. Dziewoński and Koewa (2) reported 245–246° for 2-hydroxynaphthalic anhydride.

$C_{12}H_6O_4$ . Calculated, C 67.28, H 2.82; found, C 67.45, H 3.11  
 " " 67.35, " 2.99

10.57 mg. of substance suspended in alcohol were titrated against phenolphthalein with 0.1 N NaOH. After the material was heated with a slight excess of alkali and titrated back, a total of 1.108 cc. was required. The end-point was not easily seen because of the color of the substance itself; calculated for 2 equivalents, 0.987 cc.

The substance gave a red color in alcoholic solution with ferric chloride and coupled with diazotized sulfanilic acid to give a red color.

0.075 gm. of this anhydride was heated in a solution of 0.3 gm. of NaOH in 1 cc. of water for 1.5 hours on the steam bath in an atmosphere of hydrogen. The acid which precipitated on acidification was extracted with ether and recrystallized twice from dilute ethyl alcohol with bone-black. The resulting substance melted at 257–259°. Dziewoński and Koewa (2) reported a melting point of 257° for 2-hydroxy-8-naphthoic acid.

$C_{11}H_6O_3$ . Calculated, C 70.22, H 4.28; found, C 69.95, H 4.54

The substance gave a color with  $FeCl_3$  and coupled with diazotized sulfanilic acid.

8.967 mg. of substance dissolved in alcohol were titrated against phenolphthalein with 0.1 N NaOH; found, 0.571 cc.; calculated for 1 equivalent, 0.477 cc. On being heated with excess alkali, no additional alkali was consumed.

60 mg. of the above anhydride were treated in acetone with excess diazomethane. Long slender needles were obtained from acetone, which melted at 256–257°.

$C_{13}H_8O_4$ . Calculated. C 68.42, H 3.53,  $OCH_3$  13.60  
 Found. " 68.35, " 3.70, " 13.13

This no longer gave a color with  $\text{FeCl}_3$  and did not couple with diazotized sulfanilic acid. A mixed melting point with synthetic 2-methoxynaphthalic anhydride showed no depression and it corresponded in other properties with the synthetic material. The latter was prepared by oxidation with  $\text{KMnO}_4$  in acetone solution of 3-methoxyacenaphthenequinone which was obtained according to the directions of Staudinger, Goldstein, and Schlenker (4). The anhydride was recrystallized from acetone and melted at  $256\text{--}257^\circ$ . Dziewoński and Koewa (2) and Davies, Heilbron, and Irving (3) reported a melting point of  $255^\circ$ .

$\text{C}_{11}\text{H}_8\text{O}_4$ . Calculated, C 68.42, H 3.52; found, C 68.51, H 3.33

*Decevinic Acid*—Since the details of the method of preparation and properties of this substance and its ester could not be recorded in the earlier communication (1), they are now given in detail.

20 gm. of recrystallized cevine were dissolved in a mixture of 200 cc. of  $\text{H}_2\text{SO}_4$  (1.84) and 800 cc. of water. 100 gm. of  $\text{CrO}_3$  were then carefully added with cooling to avoid appreciable rise in temperature. Vigorous evolution of gas occurred during the addition of the reagent. After standing at room temperature for an hour, the solution was refluxed for an hour. It was then cooled and the excess of chromic acid was reduced with hydrazine hydrate. The mixture was then continuously extracted with ether overnight and the resulting ether extract was decanted from a small amount of a green solid deposit. It was dried over anhydrous  $\text{MgSO}_4$  and the solvent was boiled off. The colorless liquid residue weighed approximately 10 gm. It was water-soluble and of acid character. Since repeated efforts to obtain crystalline material directly from this liquid were unsuccessful, it was treated as follows:

The mixture was heated in a flask through which a current of N was passed for an hour in an oil bath held at a temperature of  $180^\circ$ . During this heating crystalline material appeared in the melt and slow evolution of a gas was apparent. The melt was then treated with 4 volumes of ether and, after trituration in order to dissolve sticky material, the crystalline residue was collected with a little ether. It weighed 2 gm. Upon recrystallization from acetone the product melted at  $273\text{--}278^\circ$  with decomposition.



$[\alpha]_D^{25} = +47.6^\circ$  ( $c = 0.925$  in pyridine)  
 $C_{14}H_{14}O_6$ . Calculated, C 60.41, H 5.07; found, C 60.51, H 5.20  
 " " 60.45, " 5.14

0.0150 gm. of substance in 2.5 cc. of ethyl alcohol was titrated against phenolphthalein with 0.1 N NaOH; found, 1.114 cc.; calculated for 2 equivalents, 1.082 cc. No appreciable additional alkali was consumed after the substance was boiled for 2 hours with excess 0.1 N alkali.

0.200 gm. of substance was suspended in 1 equivalent of N NaOH. The end-point to phenolphthalein was reached only after addition of a 2nd equivalent or a total of 1.44 cc. of NaOH. Solution was just complete at this point. More alkali was then added until a total of 4.00 cc. had been reached. The solution was heated on the steam bath in an atmosphere of hydrogen for 1 hour and then titrated back with N HCl. Inclusive of the above 1.44 cc., a total of 2.08 cc. of NaOH was consumed; calculated for 3 equivalents, 2.16 cc. The substance,  $C_{12}H_{16}O_3$ , reported below, could readily be isolated from the hydrolysate.

*The Methyl Ester*,  $C_{16}H_{18}O_6$ —0.1 gm. of decevinic acid was dissolved in acetone and treated with an excess of diazomethane. Addition of ether to the concentrated solution gave a slight turbidity followed by crystallization. 70 mg. of rhombic crystals were obtained which melted at  $165$ – $166^\circ$ .

$C_{16}H_{18}O_6$ . Calculated. C 62.75, H 5.92, OCH<sub>3</sub> 20.26  
 Found. " 62.96, " 6.07, " 19.90  
 " " 62.73, " 6.01

The molecular weight determined by the Rast method was found to be 326; calculated, 306.14.

0.0149 gm. of the ester in 3 cc. of ethyl alcohol was titrated against phenolphthalein with 0.1 N NaOH; found, 0.478 cc.; calculated for 1 equivalent, 0.483 cc. After addition of excess 0.1 N NaOH and after refluxing for 2.5 hours an additional 0.437 cc. was consumed. The end-point was not very sharp because of an interfering color. This titration behavior was a little different from the titration in aqueous N NaOH.

0.200 gm. of the dimethyl ester was treated directly in the cold with N NaOH. As more NaOH was added, it dissolved but a point alkaline to phenolphthalein was not reached until 0.660

cc. had been added; calculated for 1 equivalent, 0.654 cc. All the ester was in solution at this point. When excess NaOH was added and the solution was heated on the steam bath for 1 hour, the alkaline decomposition reported below occurred with formation of the substance  $C_{12}H_{16}O_3$ .

*Acetyldecevinic Acid*—0.4 gm. of decevinic acid was refluxed with 10 cc. of acetic anhydride for 1 hour. After removal of excess reagent with toluene the residue was crystallized from acetone. 0.3 gm. of flat needles was obtained which melted at 169–171° with previous sintering.

The substance is soluble in dilute carbonate in the cold and gives a strong test with ferric chloride when dissolved in ethyl alcohol.

$C_{18}H_{18}O_7$ . Calculated, C 59.98, H 5.04; found, C 60.24, H 5.11

0.117 gm. of the substance was titrated directly against phenolphthalein with 1.1 N NaOH. 0.980 cc. was required; calculated for 3 equivalents, 0.996 cc. After addition of excess N alkali and heating for an hour, an additional mole was consumed as in previous cases.

*Acetyldecevinic Methyl Ester*—0.1 gm. of the above acetyl derivative was esterified with diazomethane in acetone. It crystallized readily on addition of ether to the concentrated solution. 0.06 gm. of leaflets was obtained which melted at 182–183°.

$C_{17}H_{18}O_7$ . Calculated. C 61.06, H 5.43,  $OCH_3$  9.28  
Found. " 61.03, " 5.49, " 9.11

0.0049 gm. of substance on direct titration with 1.01 N NaOH against phenolphthalein required 0.0410 cc.; calculated for 3 equivalents, 0.0436 cc.

*Decevinic Methyl Ester*—0.06 gm. of the above acetyl derivative was refluxed in 2 cc. of methyl alcohol for 2 hours. The solution crystallized on concentration. 0.021 gm. of the ester was collected which melted at 242–245°.

$C_{11}H_{14}O_4$ . Calculated. C 61.62, H 5.52,  $OCH_3$  10.60  
Found. " 61.27, " 5.60, " 7.25

0.0049 gm. when titrated against phenolphthalein with 1.01 N NaOH required 0.0315 cc.; calculated for 2 equivalents, 0.0332 cc.

*Partial Hydrolysis of the Ester,  $C_{18}H_{18}O_6$* —0.1 gm. of the ester was treated with 0.300 cc. of 1.1 N NaOH and the mixture was slightly warmed until just all in solution. It was treated with a slight excess of HCl and the material which separated was extracted with ether. The ether solution was dried over anhydrous  $MgSO_4$ , and then concentrated somewhat when crystallization occurred. 70 mg. of substance were collected which melted at  $128^\circ$ .

$C_{18}H_{18}O_6$ .	Calculated.	C 61.62,	H 5.52,	$OCH_3$ 10.61
	Found.	" 61.88,	" 5.51,	" 10.29

0.082 gm. of substance on direct titration with 1.1 N NaOH consumed 0.285 cc.; calculated for 1 equivalent, 0.255 cc. Boiling with acetic anhydride failed to form an acetyl derivative of this substance.

*Condensation of o-Phenylenediamine with Decevinic Acid*—1 gm. of decevinic acid and 0.78 gm. of o-phenylenediamine were dissolved in sufficient hot methyl alcohol. The methyl alcohol was then boiled off and the residue was heated on the steam bath for 1 hour. The crystalline material was recrystallized from a large volume of methyl alcohol. 0.48 gm. of yellow flat columns was obtained which melted at  $300-302^\circ$ . It was soluble in dilute carbonate and did not give a color test with ferric chloride.

$C_{20}H_{20}O_5N_2$ .	Calculated.	C 65.20,	H 5.47,	N 7.60
	Found.	" 65.44,	" 5.49,	" 7.85

0.125 gm. of substance on direct titration with 1.1 N NaOH required 0.315 cc.; calculated for 1 equivalent, 0.309 cc. Excess alkali was then added and the solution was heated for an hour on the steam bath. 0.542 cc. more of alkali was consumed. The end-point in this case was not sharp owing to interfering color.

*Alkaline Degradation of Decevinic Acid.* The Acid,  $C_{18}H_{16}O_6$ —2 gm. of decevinic acid were treated with a solution of 6.4 gm. of NaOH in 20 cc. of water. The solution was allowed to stand at room temperature for 2 hours and then acidified with HCl.  $CO_2$  was evolved. The insoluble material which separated was extracted with ether. The dried ether extract gave, on concentration, a residue which was crystallized from acetone-ether. 0.43 gm. of substance was obtained. After recrystallization it melted at  $150-155^\circ$  with effervescence.

$C_{13}H_{16}O_5$ . Calculated, C 61.88, H 6.39; found, C 61.92, H 6.54

The substance dissolved in alcohol did not give a color test with ferric chloride.

13.410 mg. of substance when titrated against phenolphthalein with 0.1 N NaOH consumed 1.057 cc. After addition of excess alkali and heating no more alkali was consumed; calculated for 2 equivalents, 1.064 cc.

*The Dimethyl Ester*,  $C_{15}H_{20}O_5$ —0.1 gm. of the above acid was esterified in acetone with diazomethane. The ester could not be crystallized and was distilled in a high vacuum. The distillate which was a clear viscous oil was analyzed as such.

$C_{15}H_{20}O_5$ . Calculated. C 64.24, H 7.19, OCH<sub>3</sub> 22.13  
Found. " 63.43, " 7.12, " 21.20

*The Ketolactone*,  $C_{12}H_{16}O_3$ —0.1 gm. of the above acid,  $C_{13}H_{16}O_5$ , was placed in a small sublimation apparatus under 0.1 mm. pressure. When a temperature of 180° was reached, a sticky viscous resin distilled. This was accompanied by gas evolution as evidenced by a fall in the pressure. The distillate crystallized readily from ether. After recrystallization from acetone it melted at 165–168°.  $[\alpha]_D^{25} = -50^\circ$  ( $c = 0.99$  in chloroform).

$C_{12}H_{16}O_3$ . Calculated, C 69.19, H 7.75; found, C 69.33, H 7.88

The substance was not soluble in sodium carbonate solution and the solution in alcohol did not give a color test with ferric chloride.

14.497 mg. of substance on direct titration against phenolphthalein with 0.1 N NaOH consumed 0.745 cc.; calculated for 1 equivalent, 0.697 cc. After addition of excess alkali and heating, no more alkali was consumed.

The substance did not react with diazomethane in acetone solution.

*The Phenylhydrazone*—0.025 gm. of the above ketolactone was treated with 0.05 gm. of phenylhydrazine and warmed a moment on the steam bath. The resulting melt was crystallized from ethyl alcohol. 0.01 gm. of needles was obtained which melted at 175–178°.

$C_{18}H_{22}O_2N_2$ . Calculated, C 72.46, H 7.43; found, C 72.35, H 7.40



*Hydrogenation of the Ketolactone,  $C_{12}H_{16}O_3$* —0.1 gm. of substance was hydrogenated with 0.05 gm. of platinum oxide catalyst in 3 cc. of alcohol, under 3 atmospheres pressure. After 1 hour absorption was completed at approximately 1.7 moles of hydrogen above that required by catalyst. The residue obtained after removal of the solvent crystallized nicely from ether-petroleum ether mixture but the crystals did not appear to be homogeneous and melted at 80–90°. The analytical data suggested loss of oxygen as well as hydrogenation. Since fractional crystallization did not appear to yield a homogeneous product, fractional distillation was attempted.

A second run of 0.1 gm. was combined with the first and the resulting material amounting to 0.18 gm. was fractionated in a small still (5 cm. column) of the type reported previously (5). Six approximately equal fractions were collected under 0.1 mm. pressure. The first fraction was crystalline and melted at 90–91°; found, C 73.68, H 9.30. The last fraction was semiliquid; found, C 69.96, H 9.39.

The first and second fractions were combined and recrystallized from ether. Heavy columns were obtained which melted at 97°.

$C_{12}H_{16}O_3$ . Calculated, C 74.19, H 9.35; found, C 74.50, H 9.23

The final fraction was recrystallized from ether. It melted at 65–73° with a few crystals persisting up to 90°. This was analyzed directly, since there was not sufficient of this material for both recrystallization and analysis.

$C_{12}H_{16}O_4$ . Calculated, C 68.54, H 8.63; found, C 69.09, H 8.72

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# APPLICATION TO URINE OF BANDIER AND HALD'S METHOD FOR DETERMINATION OF NICOTINIC ACID

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The evident importance of the rôle of nicotinic acid and its amide in nutrition, health, and disease has made it desirable to have practical methods for their quantitative determination in the body fluids and excretions in order to provide objective criteria for use in clinical investigation.

Numerous methods for the determination of nicotinic acid based on colorimetric reactions of the pyridine ring have been published. Karrer and Keller (1) and Vilter, Spies, and Mathews (2) based their tests on the reaction of pyridine with 2,4-dinitrochlorobenzene in the presence of an alkali hydroxide. Swaminathan (3), Shaw and Macdonald (4), and Ritsert (5) based their tests on the reaction of nicotinic acid in the presence of cyanogen bromide with aniline in either aqueous or alcoholic solution. In these procedures the methods are tedious, the color unstable, and interference from urinary pigments occurs.

Recently, Bandier and Hald (6) have described a colorimetric method based on a modification of the cyanogen bromide-aniline procedure, which they showed to be practical and rapid, and, as the color produced was stable over several hours, serial determinations were thereby facilitated. They found that *p*-methylanilino-phenol sulfate (metol, Agfa) with nicotinic acid and cyanogen bromide in aqueous solution in the presence of potassium hydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) yields a clear yellow color which is constant, stable, and directly proportional to the concentration of nicotinic acid. The same color is obtained with nicotinamide. This method is quite specific, as Bandier and Hald found no color-



tion with small amounts of pyridine, or with picolinic acid,  $\alpha$ -picoline, trigonelline, or methylpyridinium chloride, all pyridine derivatives occasionally found in the urine.

We have modified Bandier and Hald's colorimetric procedure for the quantitative determination of nicotinic acid so as to provide an index of urinary excretion of nicotinic acid, nicotinamide, and other pyridine derivatives that are related to the pellagra-preventive factor. It is not as yet known whether nicotinic acid and nicotinamide represent the total excretory forms of the pellagra-preventive factor. It is expedient, however, in view of the work of Askelöf and Holmberg (7) and Porje (8) to assume that in man the pellagra-preventive factor is probably excreted mainly as nicotinic acid and nicotinamide, the former in apparently much larger proportion.

### *Procedure*

2 ml. of the 24 hour urine specimen preserved with thymol and kept in a refrigerator are placed in a test-tube; to this are added 2.5 ml. of 2 per cent potassium hydrogen phosphate solution. The test-tube is then heated for 5 minutes in a water bath at 75-80°, after which 0.5 ml. of freshly prepared cyanogen bromide solution is added. After heating 5 minutes more in the water bath the test-tube is cooled under a tap to room temperature and 5 ml. of saturated (about 5 per cent at room temperature) fresh aqueous metol solution are then added. The maximum color develops after standing in the dark for 30 to 35 minutes. This solution is then compared in the Klett Summerson (9) photoelectric colorimeter (with Filter 42) against a blank (prepared simultaneously) containing the same amounts of cyanogen bromide, potassium hydrogen phosphate, metol, and distilled water made up to 10 ml. The entire procedure may be carried out in the test-tubes in which the colorimetric determinations are made. We have found the color to be stable for at least 6 hours, after which it slowly fades.

Correction for color and turbidity of the urine is accomplished by treating another 2 ml. sample of urine as above, except that, instead of metol, distilled water is added to make up the volume to 10 ml. The nicotinic acid value of the color as measured in this tube is subtracted from the first reading to give the actual amount of nicotinic acid. The scale of the colorimeter is calibrated by the

use of solutions of known amounts of nicotinic acid.<sup>1</sup> A satisfactory range of values for best readings is from 0.005 to 0.050 mg. of nicotinic acid. Although the scale of the colorimeter may be calibrated once and this scale factor used thereafter, it is preferable to redetermine the scale factor by means of a standard nicotinic acid solution every time a series of determinations is made, since the reagents must always be freshly prepared.

The cyanogen bromide is prepared by adding slowly 10 per cent potassium cyanide to saturated bromine water at room temperature to the point of decolorization. As the metol dissolves slowly and the solution must not be heated, the saturated metol solution is best prepared by adding small amounts to distilled water with thorough shaking. The solution should be pre-

TABLE I  
*Recovery of Added Nicotinic Acid*

Nicotinic acid of urine sample	Added nicotinic acid	Total nicotinic acid as determined	Nicotinic acid recovered	Recovery of added nicotinic acid
$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
2.9	2.0	4.8	1.9	95.0
12.8	4.0	16.7	3.9	97.5
2.5	6.0	8.8	6.3	105.0
3.3	12.0	14.5	11.2	93.3

pared in a brown bottle and used within 3 hours of preparation, as it oxidizes on standing.

The error in recovery of added nicotinic acid by the above procedure has been found to be less than  $\pm 7$  per cent (Table I).

### *Results*

The amount of nicotinic acid excreted in the urine by apparently normal subjects (resident physicians) consuming presumably an adequate general diet was found to vary from 3.4 to 10.2 mg. in 24 hours (Fig. 1). Successive urines from the same subject over 2 to 7 day periods showed variations of as much as 100 per cent.

The excretion of nicotinic acid in relation to the therapeutic response of one pellagrins has been studied. This patient, a 28

<sup>1</sup> Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

year-old colored female inebriate, when admitted to the hospital presented a pellagrous stomatitis but no clinical evidence of other

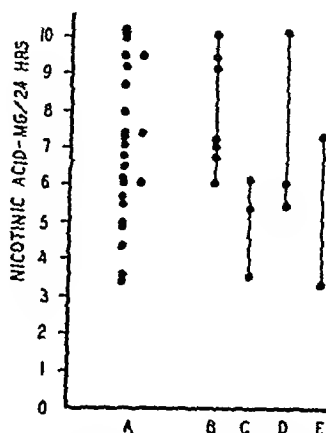


FIG. 1. Excretion of pyridine derivatives estimated as nicotinic acid in normal adults. Column A shows the range of twenty-four determinations made on thirteen subjects; Columns B, C, D, and E show the individual variations in daily excretion of four subjects.

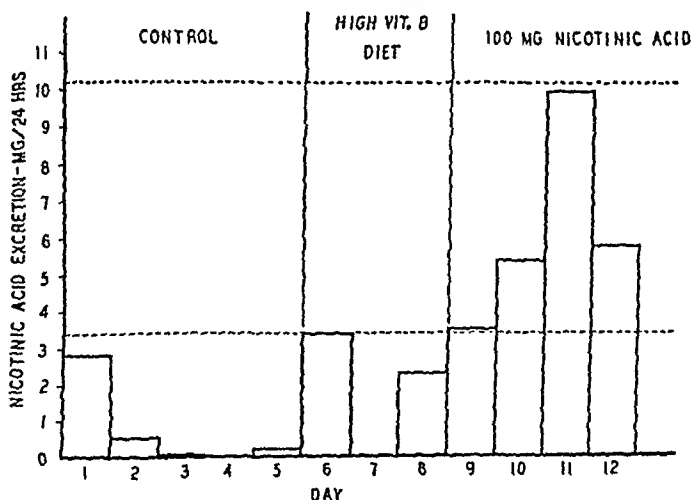


FIG. 2. Excretion of nicotinic acid in a pellagrin during a control period when the diet was low in the B vitamins, while on a high vitamin B diet, and when 100 mg. of nicotinic acid were given daily, in addition to the high vitamin B diet. The two horizontal dash lines represent the range of nicotinic acid found in the urine of thirteen normal subjects.

vitamin deficiencies. Fig. 2 shows the levels of nicotinic acid excretion when the patient was maintained with a diet deficient

in the pellagra-preventive factor (control period) (10), when she was given a diet with a high content of the vitamin B complex (11), and when this diet was supplemented with 100 mg. of nicotinic acid daily by mouth. It is seen that the patient had a low level (2.8 mg.) of nicotinic acid excretion on the 1st day of the control period, and that output decreased to zero as the diet deficient in the vitamin B complex was continued, although the stomatitis improved. Changing the diet to one high in the B vitamins was reflected by an immediate rise in urinary nicotinic acid excretion and a further increase was noted when this diet was supplemented by nicotinic acid.

#### SUMMARY

1. We have found that Bandier and Hald's method is a simple and specific procedure for the chemical determination of nicotinic acid and nicotinamide in the urine.

2. With this method the normal urinary excretion of nicotinic acid plus nicotinamide, expressed as mg. of nicotinic acid, is found to vary from 3.4 to 10.2 mg. in 24 hours.

3. Urinary nicotinic acid was low in a patient with pellagra, disappearing altogether as the diet low in the B vitamins was continued.

4. Administration of a diet high in the B vitamins, and supplementary administration of nicotinic acid, caused increases in the nicotinic acid excretion.

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## MICROESTIMATION OF URONIC ACIDS\*

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It has long been known that glucuronic acid when boiled in HCl in the presence of naphthoresorcinol forms a purple ether-soluble pigment.. The reaction, first described by Tollens (1), is given also by galacturonic acid, and has been widely used as a qualitative test for the presence of these substances. The limitations on its specificity are summarized in van der Haar's (2) monograph on the sugars.

Recently Salt (3) attempted to apply the reaction to the estimation of glucuronic acid in urine, with only partial success. His efforts were directed to the preparation of urine fractions which would yield clean violet colors, unmixed with the pinkish tones which are so troublesome in tests on whole urine. His procedure consisted in the separation of glucuronides from other urinary constituents by fractional precipitation with lead salts; the formation of the colored derivative with naphthoresorcinol, however, was performed in the classical manner, which, as will be seen, would be unlikely to yield reproducible results.

The need for a simple and reasonably accurate method of determining uronic acids in urine led us to examine the Tollens reaction itself, to see whether it might not be adapted to quantitative use by modifying the conditions under which it is carried out. It soon became evident that the conditions ordinarily employed are far from optimal for color development. However, when various factors had been adjusted so as to produce maximum amounts of pigment, the sensitivity was markedly increased and the color intensity was found to be a linear function of the concentration of uronic acid.

\* The work reported in this communication was carried out under a grant from the W. K. Kellogg Foundation.

The following factors required modification from the original form of the reaction: (1) *Time of heating with HCl*. Maximum color development required several hours, instead of the 1 minute boiling period of the classical test. (2) *Concentration of HCl*. The optimum acid concentration was 3 parts of concentrated HCl to 7 of aqueous solution, instead of equal parts of each. (3) *Concentration of naphthoresorcinol*. The intensity of color developed by a given quantity of uronic acid was found to vary with the amount of naphthoresorcinol used. However, the smaller the excess of this reagent, the cleaner were the colors obtained. The amount of naphthoresorcinol added to each test was accordingly reduced to one-tenth of the amount recommended by Tollens. (4) *Dissolution of the pigment*. The extractability of the pigment from the reaction mixture by ether was enhanced by reducing the concentration of acid prior to extraction. Other immiscible solvents (benzene, ethyl acetate, petroleum ether, chloroform) either failed to extract the pigment or yielded unsatisfactory colors.

Color intensity was measured in the Pulfrich stufenphotometer. Since the pigment has a well defined absorption maximum at 578  $m\mu$ , Filter S-57 is well suited for its measurement. Under the conditions finally adopted as standard, samples could be used containing from 10 to 40  $\gamma$  of glucuronic acid, or from 7 to 30  $\gamma$  of galacturonic acid, in a volume of 3.5 ml. or less.

#### *Details of the Estimation*

##### *Reagents Used—*

Naphthoresorcinol (C.P., Eimer and Amend), 1 per cent solution in 95 per cent ethyl alcohol. Store in a brown bottle in the refrigerator.

Ethyl ether, peroxide-free.

Sodium sulfate, anhydrous.

Hydrochloric acid, concentrated, C.P.

*Procedure for Glucuronic Acid*—Place a sample containing from 10 to 40  $\gamma$  of free glucuronic acid in 3.5 ml. of water in a centrifuge tube of 10 or 15 ml. capacity. Add exactly 0.1 ml. of naphthoresorcinol solution, followed by 1.5 ml. of concentrated HCl. Mix by rotating the tube. Place in a boiling water bath or steam bath and heat, *uncovered*, for 4.5 hours; mix by rotating

the tubes every half hour for the first 2 hours. Cool and centrifuge at 2000 R.P.M. for 10 minutes. Remove the bulk of the supernatant with a capillary pipette, avoiding disturbance of the precipitate, and dilute the remaining drop or so of fluid with about 10 times its volume of water. Extract the aqueous suspension with successive small portions of ether (about 1 ml. at a time), using a capillary pipette to mix the materials thoroughly, and transferring the violet-colored extracts to a test-tube. The total volume of pooled extracts should not exceed 6 ml. Dry the extract by adding to it a small amount of anhydrous sodium sulfate. Filter through fine filter paper (Whatman No. 44 is satisfactory) into a 10 ml. volumetric flask, using the capillary pipette to transfer the solution to the filter and washing the filter paper dropwise with as much fresh ether as the capacity of the flask will allow. With careful washing, the same funnel and paper can be used for a series of extracts. Make up to volume. Read the solutions as soon as possible in the stufenphotometer, using Filter S-57 and 20 mm. cells, against an ether blank. If there is any delay between the preparation of the solutions and their reading, protect them from light and keep in the refrigerator. With good ether, they may be kept overnight, though this is to be avoided whenever possible.

*Procedure for Galacturonic Acid*—For galacturonic acid, the working range is from 7 to 30  $\gamma$  per sample, and 2 hours on the steam bath suffice for maximum color development.

*Calculation of Results*—The color intensities developed by known amounts of glucuron<sup>1</sup> and galacturonic acid monohydrate<sup>1</sup> are shown graphically in Fig. 1. The density is that of a 20 mm. layer of solution. Equations for the conversion of density ( $D$ ) to mg. were derived empirically from these data, and are represented by the straight lines shown in Fig. 1. The equations are the following.

$$(D - 0.08)/16.7 = \text{mg. glucuronic acid (as C}_6\text{H}_{10}\text{O}_7\text{)}$$

$$(D - 0.08)/24.5 = \text{mg. galacturonic acid (as C}_6\text{H}_{10}\text{O}_7\text{)}$$

The constant 0.08 is in effect a correction for the amount of light absorption produced by the reagents alone. (Blank deter-

<sup>1</sup> Authentic specimens of these substances were kindly furnished by Dr. Walther Goebel of the Rockefeller Institute.



minations yield pale pinkish, straw-colored extracts.) Its possible variation from one batch of naphthoresorcinol to another must be controlled by determining a blank for each new lot of reagent, as the commercial c.p. grade is brown.

The range of concentration can be extended in preliminary estimations by reading very strong colors in a 10 mm. cell and doubling the value of the density thus obtained. Such a reading

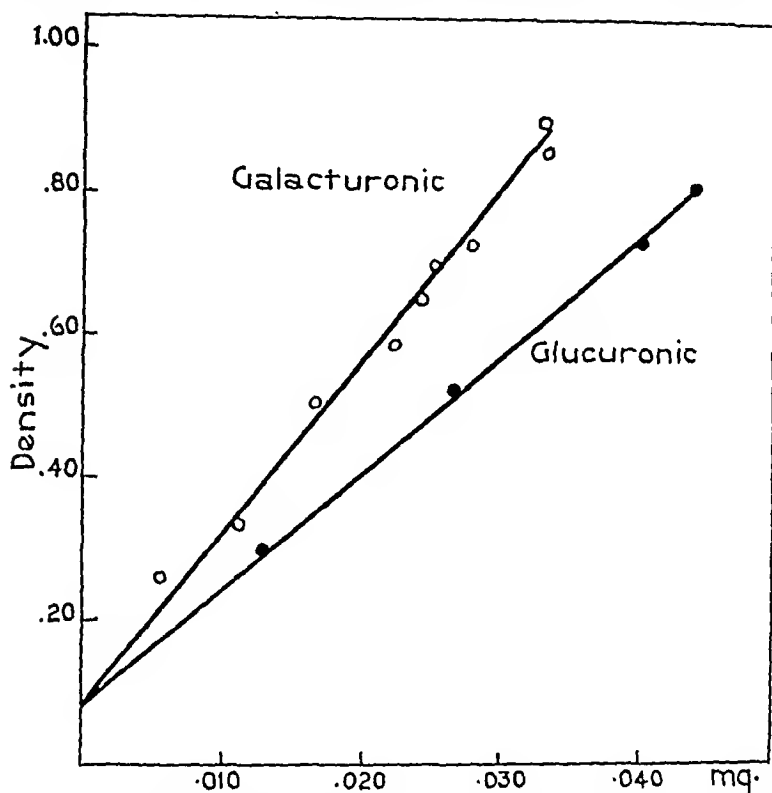


FIG. 1. The relation between the size of the sample of uronic acid and the intensity of color developed.

should be followed by a new determination with a smaller sample, calculated to fall within the specified working range, where the conversion formula is known to be valid.

A number of substances, including pentoses, interfere with the qualitative test by developing pink colors which obscure the definitive purple pigment. Such substances will of course interfere with the quantitative estimation as well, since any light absorption of extraneous origin which falls within the range of the filter

will register as glucuronic acid. For example, a pure specimen of *d*-arabinose formed a dark blue precipitate with naphthoresorcinol under the conditions described here. This precipitate yielded a rose-colored ethereal extract, with an absorption maximum in the range of Filter S-47. However, it also showed absorption in the range of Filter S-57, of the same order of intensity as had been obtained with the glucuronic acid pigment. It is therefore essential that preparations of uronic acid for estimation be free of contaminants which produce overlapping absorption.

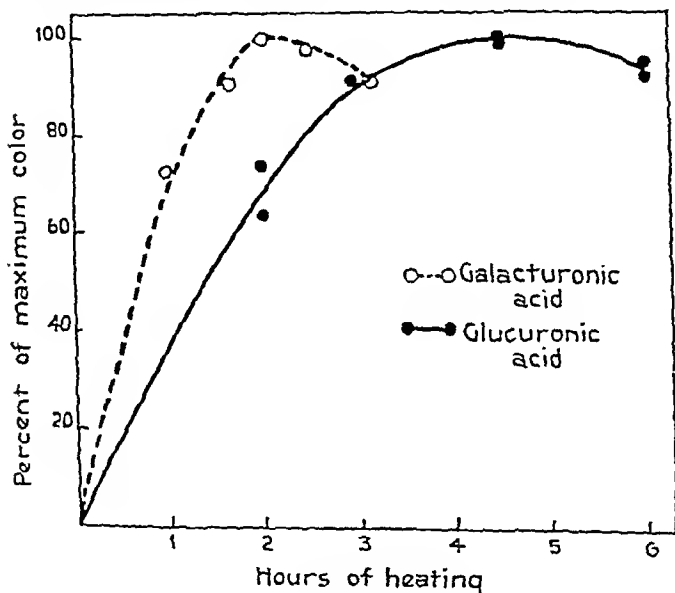


Fig. 2. The rate of color development of glucuronic and galacturonic acids.

Salt (3) has described a method of preparing suitable fractions from urine.

In an unknown preparation it is of course necessary to determine whether one is dealing with glucuronic or galacturonic acid, in order to calculate the results. This can readily be done by making use of the rate of color development as a criterion. The rates of color development of the two acids are shown in Fig. 2, from which it will be seen that they are clearly distinguishable.

The applicability of the method to a series of preparations from

urine was tested by recovery experiments, in which known amounts of glucuronic acid were added to samples of previously analyzed material. These experiments are summarized in Table I. The largest discrepancy between found and calculated values amounted to 3  $\gamma$  in 39  $\gamma$ . Duplicates as a rule agreed within 1 or 2  $\gamma$ .

### *Observations on the Reaction*

One feature of the reaction whereby the pigment is formed warrants discussion. Not only does the amount of pigment vary with the amount of naphthoresorcinol, but the color developed by a constant amount of glucuronic acid was found to be linear with the logarithm of the naphthoresorcinol concentration (see Fig. 3). From this we infer that naphthoresorcinol, besides

TABLE I

*Recovery of Known Glucuronic Acid Added to Previously Analyzed Urine Preparations*

Preparation No.	In original sample Found	In sample + 0.018 mg. known glucuronic acid	
		Calculated	Found
	mg.	mg.	mg.
Ac23	0.008	0.026	0.026
"	0.018	0.036	0.035
Sm5	0.011	0.029	0.029
"	0.021	0.039	0.036
Sm6	0.011	0.029	0.028

reacting with uronic acid, undergoes some other process which gradually reduces the amount available for pigment formation. Visible evidence of such a side reaction is to be found in the formation of a grayish precipitate in blank determinations containing only naphthoresorcinol, water, and HCl. The development of the precipitate in the blank roughly parallels, in time, the development of the deep blue uronic acid precipitate in the test. The rates of the two processes are sufficiently similar to result in effective competition between them. Hence the initial relative concentrations of naphthoresorcinol and uronic acid are significant and must be controlled carefully; that is, the size of the sample must fall within a relatively narrow range, and the amount of naphthoresorcinol used must be measured accurately

if color development is to be proportional to the amount of uronic acid present.

Another aspect of the above is to be seen in the difference in behavior between glucuronic and galacturonic acids. Galacturonic acid reacts about twice as rapidly as glucuronic acid, reaching maximum color development in 2 as against 4.5 hours. Moreover, the intensity of color developed per mg. is considerably greater for galacturonic acid, as can be seen from the conversion formulae. In the case of galacturonic acid, the corrected density must be divided by 24.5 to obtain the weight in mg., where-

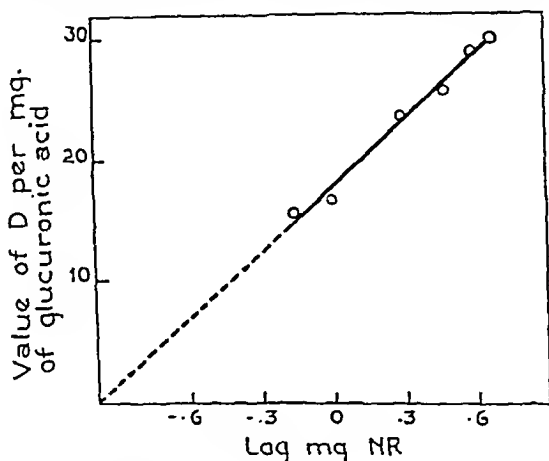


FIG. 3. The relation between the intensity of color developed by a constant amount (0.031 mg.) of glucuronic acid and the amount of naphthoresorcinol used. *D* refers to density as defined for Fig. 1.

as for glucuronic acid it is divided by only 16.7. The difference in the amount of pigment formed by the two compounds may well be related to the difference in rate of color development. In the presence of galacturonic acid, pigment formation by naphthoresorcinol takes place rapidly, before much of the reagent becomes unavailable by the side reaction. In other words, relatively more naphthoresorcinol is available for pigment formation during the reaction period as a whole in the case of galacturonic acid. It follows from these considerations, and is borne out experimentally, that uronic acid in combination must be hydrolyzed *prior* to the addition of naphthoresorcinol.

## SUMMARY

Free glucuronic or galacturonic acid can be estimated photometrically by a procedure based on the Tollens reaction.

The method is described for samples containing from 10 to 40  $\gamma$  of glucuronic acid, or from 7 to 30  $\gamma$  of galacturonic acid.

These acids can be distinguished from one another by their different rates of pigment formation with naphthoresorcinol.

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# THE REACTION OF THE ESTERS OF *dl*-LEUCINE AND OF *l*-LEUCINE ON THE RANEY CATALYST

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(Received for publication, March 9, 1940)

Catalytic reduction of esters of  $\alpha$ -amino acids to corresponding alcamines or *N*-alkylated alcamines over copper chromite catalyst was accomplished earlier in this laboratory. It was realized, however, that the nickel catalyst has a wider temperature range of activity and that therefore by changing the conditions of the reaction, the process might be controlled so that, on one hand, the desired product would be obtained and, on the other hand, the mechanism of the more complex reactions would be brought to light. Indeed, it was hoped that, by means of reduction over Raney's catalyst, conditions might be found to obtain from the esters of optically active amino acids optically active alcamines. These in their turn might serve, on one hand, for the study of the relationship of optical activity to physiological action (since derivatives of alcamines are physiologically active) and, on the other hand, as an intermediary for the assignment of the configurations of  $\alpha$ -amino acids whose configurations have not yet been established by direct chemical methods. The expectations were realized in many ways, although further refinement in procedure is possible.

The present communication deals with the results of experiments on the ethyl ester of *dl*-leucine and of *l*-leucine. The earlier experiments were performed on the *dl* form of the acid for obvious reasons.

The conditions varied with respect to temperature, duration, and proportion of catalyst.

At  $t = 135^\circ$  and an initial pressure of 2200 pounds per sq. inch the only product isolated was leucinol.

At  $t = 185^{\circ}$  and at  $200^{\circ}$  and the same initial pressure, the following products were formed: N-dimethyl-2-amino-4-methylpentane, N-dimethylleucinol, 2,5-diisobutylpiperazine, and N,N'-dimethyl-2,5-diisobutylpiperazine. In none of the experiments were all products formed but the number of them and their pro-

TABLE I

*Reduction of Leucine Ethyl Ester and Its Derivatives and of Glycylglycine Anhydride in Hydrogen over Raney's Catalyst at Varying Temperatures and at 2200 Pounds Initial Pressure*

Experiment No.	Starting substance	Weight	Duration	Temperature	Yield				
					N-Dimethyl-2-amino-4-methylpentane	2,5-Diisobutylpiperazine hydrochloride	Leucinol hydrochloride	N-Dimethylleucinol	N,N'-Dimethyl-2,5-diisobutylpiperazine
		gm.	hrs.	$^{\circ}\text{C}$ .	gm.	gm.	gm.	gm.	gm.
1	Leucine ethyl ester	2	9	70			0.6*		
2	" " "	9	22	135			3.8		
3	" " "	10	4	185		1.2		6	1.3
4	" " "	10	12	185		0.9		6	2.5
5	" " "	10	24	185	2			4.5	2.5
6	" " "	5	16	200	0.5			1.3	2.2
7	N-Dimethylleucinol	4.5	10	185	2.6				
8	Leucinol	3	12	185	1.9				
9	Leucylleucine anhydride	3	16	200					2.7
10	Glycylglycine anhydride	3	16	200					2.1†

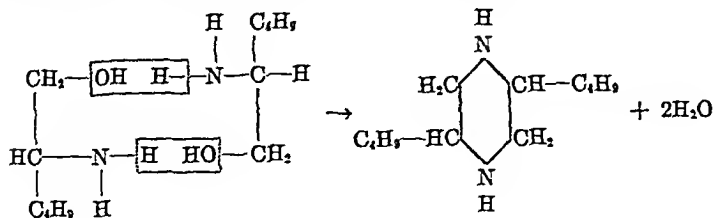
\* Leucinol.

† N,N'-Dimethylpiperazine.

portions varied with change in duration of the reaction and in temperature. The proportions are given in Table I.

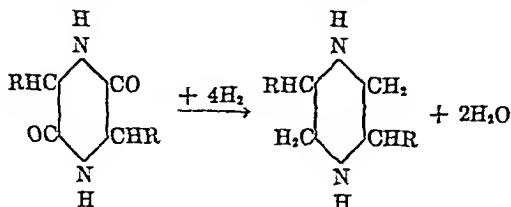
As the work progressed, the importance of the proportion of catalyst used became evident and we were encouraged to attempt the reduction of the ethyl ester of *L*-leucine at  $t = 70^{\circ}$  with a large excess of the catalyst in the hope of obtaining the optically active *L*-leucinol. This hope likewise was realized.

The mechanism of the formation of the piperazines attracted special attention. It was logical to expect that leucinol and dimethylleucinol should be the primary products of the reaction, on the basis of the experience of Paden and Adkins<sup>1</sup> and of Hill



and Adkins.<sup>2</sup> Indeed, Bain and Pollard<sup>3</sup> observed the condensation of amino alcohols into piperazines on heating over Adkins' catalyst at 235°. However, by means of Raney's catalyst under the conditions of temperature and pressure employed by us, both leucinol and dimethylleucinol failed to yield detectable quantities of piperazines, only 2-aminohexane and N-dimethyl-2-amino-hexane respectively having been isolated as products of the reaction. Hence the mechanism of the formation of piperazines required further explanation.

It was then realized that part of the leucine ester on heating in methanol solution might condense into a ketopiperazine which then might be reduced to the corresponding piperazine, which would or would not be methylated, depending on the conditions of the experiment. This expectation was fully realized, for it



was found by experiment that, on one hand, the ester heated in a sealed tube in methanol solution in the presence or in the

<sup>1</sup> Paden, J. H., and Adkins, H., *J. Am. Chem. Soc.*, **53**, 2487 (1930).

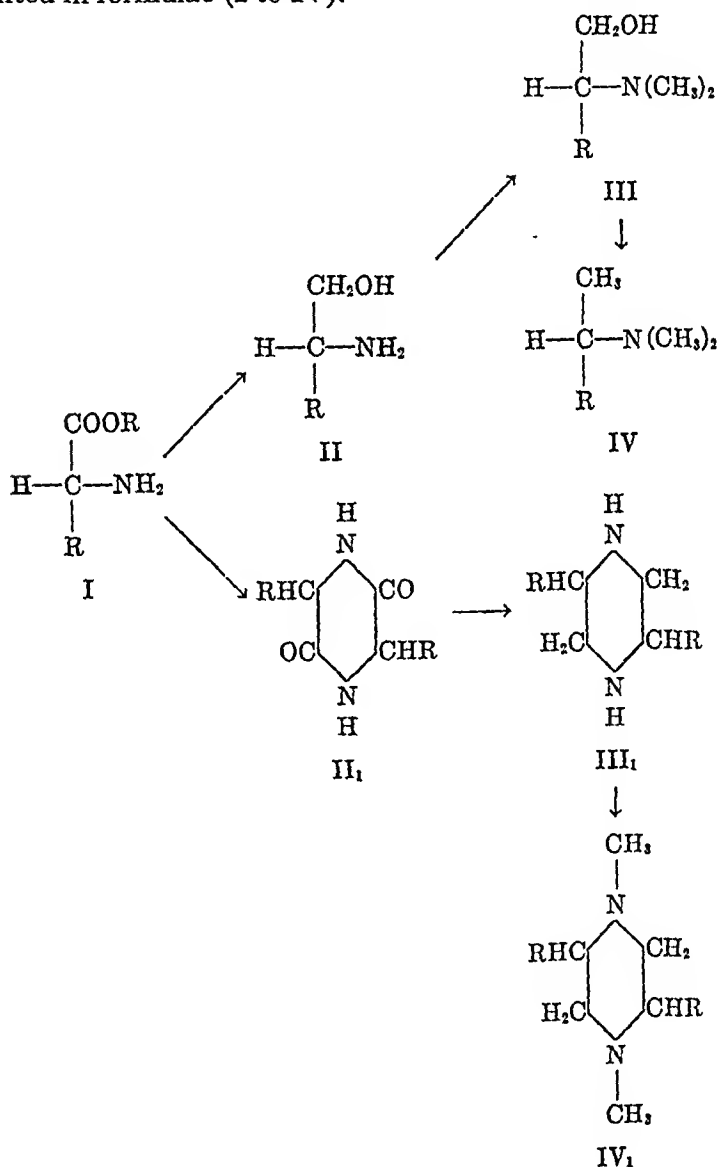
<sup>2</sup> Hill, R. M., and Adkins, H., *J. Am. Chem. Soc.*, **60**, 1033 (1938).

<sup>3</sup> Bain, J. P., and Pollard, C. B., *J. Am. Chem. Soc.*, **61**, 532 (1939).



absence of hydrogen condensed into the corresponding ketopiperazine and, on the other, that the latter was reduced to the corresponding piperazine when heated over Raney's catalyst at 185° in an atmosphere of hydrogen.

Thus at 185° and at an initial pressure of 2200 pounds of hydrogen per sq. inch two primary products are formed simultaneously, each undergoing further reaction. The course of the reaction is presented in formulae (I to IV).



## SUMMARY

1. Conditions are given for the catalytic reduction over Raney's catalyst of *dl*-leucine ethyl ester to *dl*-leucinol and for the conversion of *l*-leucine ethyl ester to *l*-leucinol.

2. Conditions are given for separation of the products formed from leucine ethyl ester over Raney's catalyst at the temperature of 185°. The products are *N*-dimethyl-2-amino-4-methylpentane, *N*-dimethylleucinol, 2,5-diisobutylpiperazine, and *N,N'*-dimethyl-2,5-diisobutylpiperazine.

3. The mechanism of formation of piperazines from esters of amino acids over Raney's catalyst is discussed.

4. A convenient procedure for the preparation of ketopiperazines is given.

## EXPERIMENTAL

*Action of Raney's Catalyst on l-Leucine Ethyl Ester in Presence of Hydrogen at 70°*—5 gm. of *l*-leucine were converted into its ethyl ester in the usual manner. Yield 3.8 gm.

4.680 mg. substance: 10.310 mg. CO<sub>2</sub> and 4.140 mg. H<sub>2</sub>O

7.081 " " : 0.539 cc. N<sub>2</sub> at 27°, *p* 761 mm.

C<sub>8</sub>H<sub>17</sub>O<sub>2</sub>N. Calculated. C 60.37, H 10.69, N 8.80

159.1 Found. " 60.07, " 10.62, " 8.67

$$[\alpha]_D^{25} = \frac{+10.8^\circ}{1 \times 0.92} = +11.7^\circ \text{ (homogeneous)}$$

4 gm. of Raney's catalyst were added to a solution of 1 gm. of *l*-leucine ethyl ester,  $[\alpha]_D^{25} = +11.7^\circ$ , in 40 cc. of absolute methanol and reduced with hydrogen at a pressure of 2200 pounds per sq. inch at 70° for 9 hours. The product was isolated as usual. B.p. 130°, *p* = 18 mm.

The substance had a composition agreeing with that calculated for *l*-leucinol.

3.485 mg. substance: 7.800 mg. CO<sub>2</sub> and 4.065 mg. H<sub>2</sub>O

4.185 " " : 0.438 cc. N<sub>2</sub> at 28°, *p* 757 mm.

C<sub>8</sub>H<sub>15</sub>ON. Calculated. C 61.47, H 12.90, N 11.95

117.1 Found. " 61.10, " 13.02, " 11.85

$$[\alpha]_D^{25} = \frac{+0.27^\circ \times 100}{1 \times 7.1} = +3.8^\circ \text{ (in methanol); } n_D^{25} = 1.4476$$

*l*-Leucinol Picrate—0.2 gm. of distilled *l*-leucinol was dissolved in 1 cc. of ether and an ether solution of 0.2 gm. of dry picric acid was added. After standing for several hours the product had completely crystallized.

A constant melting point of 120–121° was reached after recrystallization from methanol.

The substance had a composition agreeing with that calculated for the *l*-leucinol picrate.

5.276 mg. substance: 8.085 mg. CO<sub>2</sub> and 2.525 mg. H<sub>2</sub>O

4.875 " " : 0.691 cc. N<sub>2</sub> at 28°, *p* 761 mm.

C<sub>12</sub>H<sub>16</sub>O<sub>8</sub>N<sub>4</sub>. Calculated. C 41.61, H 5.26, N 16.18

346.1 Found. " 41.78, " 5.36, " 16.10

$$[\alpha]_D^{25} = \frac{+0.27^\circ \times 100}{1 \times 4.6} = +5.9^\circ \text{ (in methanol)}$$

In another experiment, 2 gm. of leucine ethyl ester,  $[\alpha]_D^{25} = +10.6^\circ$  (homogeneous), were dissolved in 80 cc. of methanol and 5.5 cc. of centrifuged Raney's catalyst (8 gm.) were added. This was reduced at 2200 pounds pressure for 9 hours at 70°.

The product distilled from a bath at a temperature of 100–110° at a pressure of 12 mm. Weight 0.6 gm.,  $n_D^{25} = 1.4478$ .

$$[\alpha]_D^{25} = \frac{+0.23^\circ \times 100}{1 \times 12} = +1.9^\circ \text{ (in methanol)}$$

4.425 mg. substance: 9.995 mg. CO<sub>2</sub> and 5.080 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>13</sub>ON. Calculated. C 61.47, H 12.90

117.1 Found. " 61.59, " 12.84

*Action of Raney's Nickel Catalyst on dl-Leucine Methyl Ester in Methanol at 135°*—9 gm. of *dl*-leucine methyl ester were dissolved in 100 cc. of methyl alcohol and reduced for 24 hours with Raney's catalyst in hydrogen at a temperature of 135° and an initial pressure of 2200 pounds per sq. inch. The catalyst was removed by filtration and the filtrate was acidified with hydrogen chloride. The acidified solution was concentrated to a semi-crystalline mass and then dissolved in 150 cc. of warm acetone. After cooling, the crystalline product was filtered. Yield 3.8 gm.

Recrystallized from methanol, the substance had the correct melting point for *dl*-leucinol hydrochloride, 160–161°,<sup>4</sup> and the following composition.

<sup>4</sup> Christman, C. C., and Levene, P. A., *J. Biol. Chem.*, 124, 453 (1938).

4.914 mg. substance: 8.505 mg. CO<sub>2</sub> and 4.495 mg. H<sub>2</sub>O  
 $C_6H_{11}ON \cdot HCl$ . Calculated. C 46.87, H 10.5  
 153.6 Found. " 47.19, " 10.3

The acetone mother liquors were concentrated, yielding a mixture of unchanged leucine methyl ester and leucinol.

*Action of Raney's Nickel Catalyst on Leucine Methyl Ester in Methanol in Presence of Hydrogen at 185°.* I. *2,5-Diisobutylpiperazine Hydrochloride*—10 gm. of the ester were dissolved in 100 cc. of methyl alcohol and reduced with hydrogen in the presence of Raney's catalyst at the temperature of 185° and initial pressure of 2200 pounds per sq. inch. The reaction product was worked up as described above. The semicrystalline mass of the hydrochloride was treated with 50 cc. of warm methyl alcohol which dissolved all the material in some experiments and left some insoluble material in other experiments. The filtrate after the removal of the insoluble substance was worked up as given in section (II). The insoluble substance was dissolved in 10 cc. of water, cooled to 0°, and treated with 10 cc. of a cold 25 per cent sodium hydroxide solution. The free base was extracted with ether and the ether extract was dried with potassium carbonate. After removal of the ether a crystalline substance was obtained which was purified by three recrystallizations from pentane. This substance melted at 79–80° and had a composition agreeing with that calculated for a dibutylpiperazine.

3.222 mg. substance: 8.590 mg. CO<sub>2</sub> and 3.790 mg. H<sub>2</sub>O  
 4.110 " : 0.50 cc. N<sub>2</sub> at 27°, p 763 mm.  
 $C_{12}H_{21}N_2$ . Calculated. C 72.72, H 13.2, N 14.1  
 198.2 Found. " 72.64, " 13.2, " 13.9

II. *N-Dimethyl-2-Amino-4-Methylpentane*—After removal of the dibutylpiperazine hydrochloride, the methyl alcohol solution was concentrated to dryness and dissolved in 50 cc. of cold water. This solution was treated with 40 cc. of a cold 25 per cent sodium hydroxide solution and the free base was extracted with ether. The ether extract was dried with potassium carbonate and the ether distilled off through a 10 inch Vigreux column, the temperature of the bath being kept below 65°.

The crude material was then fractionally distilled through a 6 inch Vigreux column at a pressure of 20 to 22 mm. The first fraction distilled at 50–60° and was redistilled under atmospheric

pressure, boiling at 110–115°. This substance had a composition agreeing with that calculated for an N-dimethylaminohexane.

4.823 mg. substance: 13.109 mg. CO<sub>2</sub> and 6.500 mg. H<sub>2</sub>O  
 $C_8H_{19}N$ . Calculated. C 74.42, H 14.9  
 129.2 Found. " 74.12, " 15.1

The substance was converted into the picrate by the procedure previously described.<sup>4</sup> M.p. 132–134°. It had the following composition.

4.504 mg. substance: 7.820 mg. CO<sub>2</sub> and 2.490 mg. H<sub>2</sub>O  
 $C_{14}H_{22}O_7N_4$ . Calculated. C 46.92, H 6.19  
 358.2 Found. " 47.34, " 6.18

*III. N-Dimethyllaucinol*—The next fraction, after removal of the N-dimethyl-2-amino-4-methylpentane, was collected between 110–120°, giving a substance which had a composition agreeing with that calculated for an N-dimethylaminohexanol.

3.008 mg. substance: 7.302 mg. CO<sub>2</sub> and 3.485 mg. H<sub>2</sub>O  
 7.709 " " : 0.652 cc. N<sub>2</sub> at 28.5°, *p* 767 mm.  
 $C_8H_{19}ON$ . Calculated. C 66.14, H 13.18, N 9.66  
 145.2 Found. " 66.19, " 12.96, " 9.66

This substance formed a picrate which melted at 105–106° in agreement with the melting point given for the picrate of *dl*-N-dimethyllaucinol. It had the following composition.

5.287 mg. substance: 8.700 mg. CO<sub>2</sub> and 2.790 mg. H<sub>2</sub>O  
 $C_{14}H_{22}O_8N_4$ . Calculated. C 44.89, H 5.98  
 374.2 Found. " 44.87, " 5.90

*IV. N,N'-Dimethyl-2,5-Diisobutylpiperazine*—After removal of the dimethyl-2-amino-4-methylpentane and the dimethyllaucinol the still residue was separated into two fractions, the first one distilling between 120° and 150° at 22 mm. and composed of a mixture of N-dimethyllaucinol and N,N'-dimethyldiisobutylpiperazine. The proportion of each in this fraction was calculated from the carbon and hydrogen values.

The second fraction distilled at 150–155° at a pressure of 1 to 2 mm. This substance contained from 72 to 73 per cent carbon, indicating the presence of some dimethyllaucinol. Accordingly, a picrate was made of this fraction by dissolving the product in hot

methyl alcohol and adding a hot methyl alcohol solution of picric acid. The picrate was insoluble in the hot methanol, thus separating it from the soluble picrate of dimethyllleucinol. The picrate dissolved in hot absolute ethanol and settled out again on the addition of ether. It had a melting point of 255–257° and a composition agreeing with that calculated for the picrate of an N,N'-dimethyldiisobutylpiperazine.

5.910 mg. substance: 9.890 mg. CO<sub>2</sub> and 2.720 mg. H<sub>2</sub>O  
 $C_{11}H_{18}N_2 \cdot C_{12}H_8N_4O_{14}$ . Calculated. C 45.61, H 5.3  
 684.3 Found. " 45.63, " 5.2

This picrate was then decomposed according to the directions given for decomposition of the picrate of N-dimethyllleucinol,<sup>4</sup> giving a product which distilled at 80–85° at 0.3 to 0.5 mm. pressure. The substance had a composition agreeing with that calculated for an N,N'-dimethyldibutylpiperazine.

3.303 mg. substance: 9.005 mg. CO<sub>2</sub> and 3.990 mg. H<sub>2</sub>O  
 5.022 " " : 0.549 cc. N<sub>2</sub> at 26°, p 754 mm.  
 5.311 " " : 5.311 cc. 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>  
 $C_{11}H_{18}N_2$ . Calculated. C 74.34, H 13.4, N 12.39, CH<sub>3</sub>(N) 13.22  
 226.3 Found. " 74.34, " 13.5, " 12.38, " 12.73

*Action of Raney's Nickel Catalyst on Dimethyllleucinol in Methanol at 185°*—Dimethyllleucinol (4.5 gm.) was dissolved in 100 cc. of methanol and reduced for 10 hours with 3 gm. of Raney's nickel, at a temperature of 185° and an initial pressure of 2200 pounds. The product was worked up as the free base in the manner described previously. The colorless liquid *completely* distilled at 60–70° under 20 mm. pressure and then redistilled at 110–115° under atmospheric pressure. Yield 2.6 gm.

The picrate of this substance melted at 132–134°. The substance thus had the properties of N-dimethyl-2-amino-4-methylpentane.

*Synthesis of N,N'-Dimethylpiperazine by Reduction of Glycylglycine Anhydride*—3.0 gm. of glycylglycine anhydride were dissolved in 30 cc. of dry methanol. To the solution were added 5.0 gm. of Raney's catalyst and reduction was carried out at 200° as described. The filtrates from the catalyst and the washings were combined and acidulated with hydrogen chloride gas until acid to Congo red and were then concentrated to a thick syrup.

The residue was taken up in 10 cc. of water and an aqueous solution of sodium picrate was added to it. The yield was nearly quantitative, 9.5 gm. of picrate (1.9 gm. of base). After recrystallization, the melting point was 280°.

The substance had the following composition.

5.822 mg. substance:	8.085 mg. CO <sub>2</sub> and 1.990 mg. H <sub>2</sub> O
4.620 " "	: 0.797 cc. N <sub>2</sub> at 27°, p 750 mm.
C <sub>18</sub> H <sub>20</sub> O <sub>14</sub> N <sub>8</sub> .	Calculated. C 37.75, H 3.55, N 19.57
572.2	Found. " 37.91, " 3.82, " 19.37

*Synthesis of N,N'-Dimethyl-2,5-Diisobutylpiperazine by Reduction of dl-Leucylleucine Anhydride*—3.0 gm. of leucylleucine anhydride and 5 gm. of Raney's catalyst were treated as in the previous experiment. The yield of the picrate was 9.6 gm. (2.82 gm. of base). M.p. 256–258°.

The substance had the following composition.

5.410 mg. substance:	8.995 mg. CO <sub>2</sub> and 2.595 mg. H <sub>2</sub> O
5.615 " "	: 0.812 cc. N <sub>2</sub> at 26°, p 762 mm.
C <sub>26</sub> H <sub>34</sub> O <sub>14</sub> N <sub>8</sub> .	Calculated. C 45.59, H 5.30, N 16.35
684.3	Found. " 45.32, " 5.36, " 16.20

*Condensation of dl-Leucine Methyl Ester to Its Anhydride in Hydrogenation Apparatus in Absence of Catalyst*—This experiment was undertaken to test the hypothesis of piperazine formation from leucine methyl ester through the intermediate formation of ketopiperazine. 4 gm. of dl-leucine methyl ester were dissolved in 15 cc. of dry methanol and placed in the hydrogenation apparatus without catalyst for 9 hours at an initial pressure of 1900 pounds per sq. inch and a temperature of 150°. The apparatus was allowed to cool for 10 hours. The partially crystalline reaction product was concentrated under reduced pressure to a white crystalline mass. Yield 2.6 gm.; m.p. 268–270° after recrystallization from 98.5 per cent ethanol.

The substance had the following composition.

4.295 mg. substance:	10.000 mg. CO <sub>2</sub> and 3.790 mg. H <sub>2</sub> O
4.300 " "	: 0.469 cc. N <sub>2</sub> at 27°, p 759 mm.
C <sub>12</sub> H <sub>22</sub> O <sub>2</sub> N <sub>2</sub> .	Calculated. C 63.66, H 9.81, N 12.38
226.2	Found. " 63.49, " 9.87, " 12.38

*Condensation of dl-Leucine Methyl Ester to dl-Leucylleucine Anhydride*—3 gm. of dl-leucine methyl ester were taken up in 5

cc. of dry methanol and heated in a sealed tube at 150° for 9 hours. The partially crystalline reaction product was concentrated under reduced pressure to a crystalline mass. Yield 2.0 gm.; m.p. 268–270° after recrystallization from ethanol.

The substance had the following composition.

4.926 mg. substance: 11.505 mg. CO<sub>2</sub> and 4.300 mg. H<sub>2</sub>O

4.480 " " : 0.484 cc. N<sub>2</sub> at 24°, *p* 759 mm.

C<sub>12</sub>H<sub>22</sub>O<sub>2</sub>N<sub>2</sub>. Calculated. C 63.66, H 9.81, N 12.38

226.2 Found. " 63.69, " 9.76, " 12.40

In a similar way 1.0 gm. of *L*-leucine methyl ester,  $[\alpha]_D^{26} = +16.7^\circ$ , yielded 0.7 gm. of the active ketopiperazine, m.p. 271°.

4.313 mg. substance: 10.100 mg. CO<sub>2</sub> and 3.790 mg. H<sub>2</sub>O

4.600 " " : 0.502 cc. N<sub>2</sub> at 26°, *p* 759 mm.

C<sub>12</sub>H<sub>22</sub>O<sub>2</sub>N<sub>2</sub>. Calculated. C 63.66, H 9.81, N 12.38

226.2 Found. " 63.85, " 9.90, " 12.48

$$[\alpha]_D^{25} = \frac{-0.45^\circ \times 100}{1 \times 16} = -2.81^\circ \text{ (in methanol)}$$





# THE STRUCTURE OF DEXTRAN SYNTHESIZED FROM SUCROSE BY BETACOCOCCUS ARABINOSACEUS, ORLA-JENSEN

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The chemical structure of the dextran produced by the action of bacteria of the *Betacoccus arabinosaceus* (*Leuconostoc mesenteroides*) group on sucrose has been recently studied by several authors. The results of Fowler, Buckland, Brauns, and Hibbert (1) indicate that the dextran formed by a strain of *Leuconostoc mesenteroides*<sup>1</sup> is a polymer of a pentaglucofuranose anhydride

<sup>1</sup> Since there is no general agreement as to the delimitation of the species *Betacoccus* (*Leuconostoc*) *arabinosaceus*, *Betococcus mesenteroides*, and *Betacoccus dextranicus*, these specific names are of little value in identifying individual organisms. The identity or non-identity of two strains of dextran-forming heterofermentative lactic acid bacteria can at present be recognized only by reference to specific characters, of which the most useful appear to be the abilities to ferment arabinose, xylose, and raffinose and to form definite capsules. The organism used by Fowler, Buckland, Brauns, and Hibbert (1) ferments both arabinose and xylose but not raffinose; no capsules were observed. The organism used by Fairhead, Hunter, and Hibbert (2) is not described; but if these authors follow the Hueker and Pederson classification, the specific name *dextranicus* indicates an organism unable to ferment either arabinose or xylose. Peat, Schlüchterer, and Stacey (3) also failed to describe the organism with which they worked. Since, however, the culture was obtained from Professor A. J. Kluyver (Delft) and is referred to as *Betococcus arabinosaceus haemolyticus* (*Leuconostoc dextranicum*) it almost certainly has the following characteristics: arabinose and raffinose are fermented, xylose may or may not be fermented, capsules are not formed, hemolysis occurs. The organism we have used ferments arabinose but not xylose or raffinose; capsule formation has never been observed. Therefore, it differs from the organisms referred to above in respect to the ability to ferment either xylose or raffinose. It should be emphasized, however, that all of the dextran-forming heterofermentative lactic acid bacteria are or appear to be very closely related.

in which three of the linkages between the glucose units are of the 1,6 type, while the remaining two are either 1,4 or 1,6. No data were given with regard to the molecular weight of the dextran.

The dextran obtained by Fairhead, Hunter, and Hibbert (2) through the action of another strain (referred to as *Leuconostoc dextranicus*<sup>1</sup>) on sucrose yielded 2,3,4-trimethylmethylglucoside and about 10 per cent dimethylmethylglucoside as the sole products of hydrolysis of the methylated polysaccharide, but no tetramethylmethylglucoside was obtained. Since no evidence of the presence of any end-groups has been found, these authors conclude that they are not dealing with a short chain polymer but rather with a linear polymer in which the terminal units of one chain are linked with members of adjacent chains.

Peat, Schlüchterer, and Stacey (3) in their investigation of the dextran produced by a third strain<sup>1</sup> of *Betacoccus* report a small amount of tetramethylglucose (0.23 per cent of the weight of methylated dextran) in the hydrolysis product of the methylated derivative. This end-group fraction indicates a chain length of about 550 glucose units. The molecular weight determination by osmotic pressure measurements gave a value corresponding to 200 glucose units.

The present investigation of the dextran produced from sucrose by a strain of *Betacoccus arabinosaceus*<sup>1</sup> isolated from soil confirms the results of Fowler, Buckland, Brauns, and Hibbert (1) in that the main hydrolytic fraction of the methylated dextran is 2,3,4-tetramethylmethylglucoside. The quantity of dimethyl- and tetramethylglucose obtained from the hydrolysis products of the methylated dextran, however, was much lower than that obtained by these authors. The small amount of tetramethylglucose (end-group fraction) suggests that the polysaccharide is made up of chains of anhydroglucose units joined uniformly by glucosidic linkages between the 1st and 6th carbon atoms to form a terminated chain. The molecular weight of the dextran as determined by Staudinger's viscosity method on the methylated product with  $K_{\eta} = 10^{-3}$  (4) was 11,700. Since, however, it is not certain whether this method can be applied for the molecular weight determination of this polysaccharide, the value obtained cannot be regarded as reliable. It is considered, therefore, that

the value  $2600 \pm 50$ , obtained by the ultracentrifuge (sedimentation equilibrium method), gives a more trustworthy result. The dextran is insoluble in water, but dissolves in alkaline solution. Its specific rotation  $[\alpha]_D$  in 1 N sodium hydroxide was  $+184^\circ$ . The high positive rotation of the dextran and its downward mutarotation during hydrolysis indicate that the 1,6-glucosidic linkage in the dextran has the  $\alpha$  configuration.

#### EXPERIMENTAL

The dextran was prepared by inoculating the organism into a medium of the following composition: sucrose 600.0 gm., yeast extract 15.0 gm.,  $K_2HPO_4$  30.0 gm.,  $MgSO_4 \cdot 7H_2O$  1.2 gm.,  $(NH_4)_2SO_4$  3.6 gm., distilled water 6 liters.

The pH of the medium was 7.6. After being incubated for 6 days at  $28^\circ$  the fermented medium became highly viscous and had a reaction of pH 4.4. The dextran was then precipitated by the addition of an equal volume of 95 per cent alcohol, filtered, washed with dilute alcohol, and dried *in vacuo* at  $100^\circ$ . The dextran was insoluble in water and did not reduce Fehling's solution. The ash content of an anhydrous sample was 0.9 per cent, nitrogen 0.1 per cent.

*Specific Rotation*— $[\alpha]_D = +184^\circ$  (in 1 N sodium hydroxide,  $c = 1$ )

*Analysis*<sup>2</sup>— $(C_6H_{10}O_5)_n$ . Calculated. C 44.4, H 6.2

Found. " 44.1, " 6.5

*Hydrolysis of Dextran and Identification of Glucose*—1 gm. of the dextran was hydrolyzed for 4 hours under a reflux condenser with 2.5 per cent sulfuric acid, neutralized with sodium hydroxide, and diluted. The reducing value determined on the neutralized solution by oxidation with ferricyanide and titration with ceric sulfate (5) was 94 per cent calculated as glucose. An osazone was prepared from the remainder of the solution by treatment with phenylhydrazine hydrochloride and sodium acetate according to Mulliken (6). The osazone separated out as a yellow precipitate when heated on a boiling water bath for 5 minutes. It was identified by its melting point and by the shape of its crystals under the microscope as glucosazone. Mannose was not present.<sup>3</sup> The

<sup>2</sup> Calculated on an ash-free basis.

<sup>3</sup> Under the conditions employed a nearly white precipitate of mannose phenylhydrazone forms within less than half a minute.

absence of fructose was shown by the negative Seliwanoff reaction carried out on the hydrolyzed solution according to Roe (7). When the polysaccharide was distilled with 12 per cent hydrochloric acid and the distillate treated with thiobarbituric acid, no precipitate was formed. This showed the absence of pentose or uronic acid units in the polysaccharide.

In order to determine the direction of mutarotation during hydrolysis of the dextran, 1 gm. of the material was dissolved in 100 cc. of 1 N hydrochloric acid by heating on a steam bath in a flask supplied with a reflux condenser. The rotation of cooled portions of the solute was determined at several time intervals. The specific rotation  $[\alpha]_D$  of the first portion determined after about 15 minutes was  $+154^\circ$ . The rotation of the successive portions diminished gradually with time until after 6 hours it reached  $+54^\circ$ , and then remained constant. This value approximately agrees with the rotation of an equilibrium mixture of  $\alpha$ - and  $\beta$ -glucose, which is  $+52.5^\circ$  in water. The downward mutarotation during hydrolysis indicates the  $\alpha$  configuration of the glucose units in the polysaccharide.

*Acetylation of Dextran*—4 gm. of the powdered dextran were soaked for 30 minutes in 25 cc. of acetic acid through which chlorine gas had been previously bubbled for 1 minute. 40 cc. of acetic anhydride, through which sulfur dioxide was passed for 1 minute, were then added, and the mixture shaken for several hours with a mechanical shaker. It was then kept at  $80^\circ$ , with occasional shaking for 2 hours. The insoluble fraction was centrifuged off and the supernatant liquid poured into a large excess of cold water. The resulting precipitate was washed with water and dried *in vacuo* at  $80^\circ$ .

*Specific Rotation*— $[\alpha]_D = +190^\circ$  (in chloroform,  $c = 1$ )

*Analysis*— $(C_6H_7O_5(CH_2CO)_3)_n$ . Calculated.  $CH_2CO$  44.8  
Found. " 44.8

*Regeneration of Dextran from Its Triacetate*—The triacetate was deacetylated by allowing it to stand overnight with 50 per cent aqueous methyl alcoholic 3 per cent potassium hydroxide. The mixture was then acidified with acetic acid, 2 volumes of 95 per cent alcohol were added, and the resulting precipitate washed

with more alcohol and then dried. The regenerated dextran differed from the original in that it was soluble in water.

*Specific Rotation*— $[\alpha]_D = +180^\circ$  (in water,  $c = 1$ )

*Methylation of Dextran*—20 gm. of the dextran were ground to a fine powder, placed in 100 cc. of water, and methylated at  $50^\circ$  with 200 cc. of methyl sulfate and 400 cc. of 30 per cent sodium hydroxide over a 4 hour period. At the end of that time the solution was heated to  $100^\circ$ ; the methylated polysaccharide separated out. After three methylations (of which the last one was conducted in the presence of acetone) a product was obtained with a methoxyl content of 36 per cent. The partially methylated polysaccharide was further methylated according to the modification of Fowler, Buckland, Brauns, and Hibbert (1) of Muskat's method (8) by dissolving the partially methylated product in anisole and then treating with liquid ammonia, sodium, and methyl iodide.

*Specific Rotation*— $[\alpha]_D = +214^\circ$  (in chloroform,  $c = 1$ )

*Analysis*— $(C_6H_7O_2(OCH_3)_3)_n$ . Calculated. OCH<sub>3</sub>, 45.6  
Found. " 45.0

*Hydrolysis of Methylated Dextran and Separation of Cleavage Products*—10 gm. of the methylated polysaccharide were permeated with 50 cc. of glacial acetic acid by being kept in a flask for an hour under reduced pressure. 100 cc. of 5 per cent hydrochloric acid were added and the mixture kept on a steam bath for 12 hours, at which time only a part of the polysaccharide went into solution. More acetic acid, hydrochloric acid, and water were added to make a 50 per cent aqueous acetic acid solution containing 4 per cent concentrated hydrochloric acid and the mixture was then kept at  $100^\circ$  for 12 more hours. At the end of that period the methylated polysaccharide had dissolved. The acid was partially neutralized with barium carbonate and the remaining acetic acid removed by vacuum distillation with addition of water as required. The residue was extracted by a mixture of alcohol and benzene. The resulting syrup, weighing 6.5 gm. and containing the trimethyl- and tetramethylglucoses, was dissolved in 45 cc. of water and separated according to the procedure of Bell (9) by extracting the aqueous solution of the mixture with

chloroform. The chloroform solution was evaporated to dryness and extracted with boiling petroleum ether. The extract after evaporation of the petroleum ether yielded 0.27 gm. of 2,3,4,6-tetramethylglucose.

*Specific Rotation*— $[\alpha]_D = +81.5^\circ$  (in water,  $c = 1$ )

*Analysis*— $C_6H_8O_2(OCH_3)_4$ .    Calculated.  $OCH_3$ , 52.6  
    Found.                    "    52.2

The aqueous extract containing the trimethylglucose was evaporated to a syrup and boiled for 12 hours under a reflux condenser with 200 cc. of methyl alcohol containing 1.5 per cent hydrogen chloride. The solution was neutralized with silver carbonate and filtered. The filtrate was clarified with charcoal, filtered again, and evaporated to a syrup. The weight of the syrup was 3.92 gm. Its methoxyl content was 51.9 per cent, which corresponds to that of trimethylmethylglucoside (calculated for  $C_6H_8O_2(OCH_3)_4$ , 52.6). The syrup began to crystallize after it was distilled *in vacuo* (0.1 mm. pressure), and kept standing for several days. The semicrystalline material was pressed on a porous glass plate and recrystallized from petroleum ether.

*Specific Rotation*— $[\alpha]_D = -20.2^\circ$  (in water,  $c = 1$ )

*Analysis*— $C_6H_8O_2(OCH_3)_4$ .    Calculated.  $OCH_3$ , 52.6  
    Found.                    "    52.3

Reported  $[\alpha]_D$  values for 2,3,4-trimethyl- $\beta$ -methylglucoside are  $-20.57^\circ$  (1) and  $-21^\circ$  (3).

The residue, containing the barium salts, after extraction with benzene, was extracted with ethyl acetate. After evaporation of the ethyl acetate 0.29 gm. of syrup was obtained. Its methoxyl content was 30.3 per cent, corresponding to that of dimethylglucose (calculated for  $C_6H_{10}O_4(OCH_3)_2$ , 29.8).

*Molecular Weight of Dextran*—The molecular weight was determined on the methylated dextran according to Staudinger's viscosity method. The specific viscosity of a 0.4 per cent solution of the methylated derivative was 0.29. This corresponds to a molecular weight of 14,800 for the methylated dextran, according to Staudinger's formula with  $K_m = 10^{-3}$  (4), and 11,700 for the dextran. However, since it was not known whether the constant  $K_m = 10^{-3}$  could be used for this polysaccharide, the viscosity method for determination of the molecular weight cannot be regarded as reliable.

The molecular weight was also determined by the sedimentation equilibrium method with the air-driven top ultracentrifuge as developed by McBain (10). The molecular weight of the methylated dextran obtained by this method was  $3275 \pm 50$ . This corresponds to a molecular weight of  $2600 \pm 50$  for the dextran. The determinations with various centrifugal forces gave the same molecular weights, indicating that the methylated polysaccharide is essentially homogeneous.

#### SUMMARY

The structure of the dextran synthesized by the action of a strain of *Betacoccus arabinosaceus* (*Leuconostoc mesenteroides*) on sucrose was studied. On treatment of this polysaccharide with acid, glucose was obtained as the sole product of hydrolysis. Its positive rotation and the downward mutarotation during hydrolysis suggest an  $\alpha$  configuration of the anhydroglucose units in the molecule.

On methylation and subsequent hydrolysis of the dextran 2,3,4-trimethyl- $\beta$ -methylglucoside was obtained as the main product of hydrolysis, and a small amount of an end-group (tetramethylglucose) was isolated. This indicates that the polysaccharide consists of  $\alpha$ -glucopyranose units linked through the 1st and 6th carbon atoms to form terminated chains.

The molecular weight of the dextran determined on the methylated derivative by the Staudinger viscosity method was 11,700. A value of  $2600 \pm 50$  was obtained for its molecular weight with the ultracentrifuge by the sedimentation equilibrium method.<sup>4</sup>

The authors wish to express their gratitude to Professor J. W. McBain for the use of the ultracentrifuge and to Mr. L. H. Perry for carrying out the molecular weight determinations.

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<sup>4</sup> Recently molecular weight determinations were carried out with the air-driven top ultracentrifuge on fully methylated starch. A value of 2750 was obtained, which is about 10 times smaller than the molecular weight determined by other investigators (11) using viscosity, osmotic, and ultracentrifugal methods. In view of this discrepancy, there exists considerable uncertainty as to the interpretation of the molecular weight reported for the bacterial polysaccharide.



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## THE COMPOSITION OF DOLPHIN MILK\*

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Values found in the literature (1-8) for the composition of cetacean milk were based on specimens collected several hours after the animals had been killed. The samples may have been contaminated with sea water, blubber oil, blood, or other body fluids, and some may have been poorly preserved; Clowes (2) has adequately discussed these points. The milk analyses reported in this paper are significant because they were obtained on uncontaminated samples from living animals.<sup>1</sup> Data will be presented on the major constituents of milk obtained from three live bottlenose dolphins (*Tursiops truncatus*) and, in addition, from one spotted dolphin (*Prodelphinus plagiodon*) 1.5 hours after it had been harpooned.

### EXPERIMENTAL

*Collection of Material*—The milk samples were taken from the captive dolphin suspended above the water in a sling. The

\* Part of the expense of this project was defrayed from a grant-in-aid of the Committee on Research in Endocrinology of the National Research Council and from the Biological Grant from the Rockefeller Foundation to the University of Chicago.

† The authors are listed alphabetically because the work was carried out by them as a group.

<sup>1</sup> The collection of this material was made possible through the wholehearted cooperation of the scientific and technical staffs of the Marine Studios, Marineland, St. Augustine, Florida. The location of the Marine Studios is ideal for the capture of dolphins; the trained personnel handles these animals without injuring or unduly exciting them. The efficient service of the Eastern Air Lines and the Air Express of the Railway Express Agency was an indispensable link in the transportation of the samples from St. Augustine or Jacksonville to the laboratories in Chicago.

nipples and surrounding region were carefully dried and protected from dripping water with towels. The milk was expressed by firmly kneading the entire length of the mammary gland toward the nipple until the flow almost ceased. The milk was white in appearance with a yellow tinge and a creamy consistency; it had a fishy odor when freshly drawn and the taste was oily and lacked sweetness. No change in consistency or appearance of the sample was observed during milking. A portion of the sample was used for osmotic pressure determinations which were carried out at the Marine Studios. After a small amount of formalin had been added as a preservative, the remainder of the sample was chilled and shipped to Chicago in the special refrigerated containers described in a previous paper (9).

*Chemical Methods*—The following determinations were made on the milk: water, total nitrogen (and in some cases non-protein nitrogen), fat, sugar, sodium, potassium, calcium, magnesium, chloride, inorganic phosphorus, pH, and osmotic pressure. All analyses were made in duplicate. The chemical methods were the same as those used for the dolphin blood (9), except that calcium and magnesium were determined on ashed samples of milk. The details followed in this procedure were the same as those reported for tissue analyses by Eichelberger and Bibler (10). The non-protein nitrogen was estimated by the modified method of Erickson, Stoner, and Macy (11). In addition, total fat was determined by both the Babcock and extraction methods, and the sugar by the Miller and Van Slyke method (12).

### Results

In Table I, the analytical results obtained from the analyses of milk from three live dolphins, Nos. 2, 4, and 5,<sup>2</sup> and one harpooned spotted dolphin, A, are given. The length of the time since parturition was unknown except for Dolphin 5, which was nearing the end of lactation. The calf following her was feeding on mullet and attempting to nurse at frequent intervals. Although the cow was milked a number of times, the sample of milk

<sup>2</sup> Both milk and blood samples were obtained from these three dolphins, and the numbers designating them correspond to those used in the previous communication (9). Milk and blood from Dolphin 4 were taken simultaneously.

TABLE I  
Concentration of Constituents of Dolphin Milk

The units are expressed per liter.

Milk	Sp. gr.	H <sub>2</sub> O	Total N <sub>2</sub>	Non-protein N	Total protein	Fat	Sugar	pH	Cl	Inorganic P	Na	K	Ca	Mg	Osmotic pressure
	gm. per cc.	gm.	gm.	gm.	gm.	gm.	gm.		m.eq.	mM	m.eq.	m.eq.	m.eq.	m.eq.	mM NaCl per l. H <sub>2</sub> O
Dolphin 2*	1.018	713.7	16.74	1.31	96.4	167	7.73	6.63	70.3	6.12	44.6	32.2	38.8	6.10	104.3
" 4*	1.020	674.2	12.40			148		7.13	51.6		73.5	15.3	30.2	5.18	164.8
" 5*, †	1.010	755.5	19.14	1.20	111.1	108	3.88		70.4		88.7	15.3	35.0	7.64	
" A†	1.013	680.8	16.16	1.09	94.2	180	6.30	6.50	64.0	10.8	42.7	43.4	38.6	5.14	170.8
Reindeer (13, 14)					111.1	187									
		682.0			104.0	171	20.8								
Rabbit (15, 16)		733.9			103.8	121.7	18.4								
					114.0	121.0	18.0								
Dog (17)		769.0			87.4	100	27.4								
		747.0			90.2	111	27.3								
Human (14)		874.1			22.8	37.6	62.9								

The figures in parentheses refer to the bibliography.

\* *Tursiops truncatus*.

† Animal near end of lactation.

‡ *Prodelphinus plagiodon*, harpooned.

obtained was always small. The points of interest in the data are as follows: (1) All of the milks were rich in fat and protein and low in lactose; (2) the sums of the inorganic base concentrations were 121.7, 133.2, and 129.8 milliequivalents per liter in Dolphins 2, 4, and A, respectively, and in Dolphin 5 (near the end of the lactation period) the total base concentration was 146.6 milliequivalents per liter; and (3) the percentage of solid matter in the milk of Dolphin 5 was 24.45, while 31.02, 28.63, and 32.58 per cent were found respectively in the milk of Dolphins A, 2, and 4. Because of the differences in the composition of the milk from the different animals of the same species, mean values are not presented.

#### DISCUSSION

Many of the values in the analytical data of this study of dolphin milk differed from those reported by previous investigators, while some were comparable. The differences may be attributed to various factors, among which are the following: (1) the milk was obtained from different species, (2) the milk was obtained from animals in different stages of lactation, and (3) the fact that the milk analyzed was obtained from living dolphins. Although variations were found in the composition of milk from the different animals of the same species, all of the specimens were high in proteins (ranging from 94.2 to 111 gm. per liter), high in fat (ranging from 108 to 180 gm. per liter), and low in lactose (ranging from 3.9 to 7.7 gm. per liter). Thus, this rich milk supplies the necessary constituents required by the calf for its rapid rate of development.

The most apparent similarities in the protein and fat content of milk from other mammals are found in the reindeer, rabbit, and dog (Table I). All of these, therefore, have high caloric values. The dolphin milk, however, has a low lactose content, which is its most differentiating factor. These findings agree with those of many other whale milk analyses (2-8). The dolphin milk is obviously dissimilar to human milk.

Some fluctuation was found in the mineral constituents of the dolphin milk, the largest variations being in the sodium and potassium concentrations. Although the individual concentration of sodium and potassium varied, their sums were quite constant;

for example, in Dolphins 2, 4, and A, the sums were 76.8, 89.0, and 86.2 milliequivalents per liter. In Dolphin 5, the animal near the end of lactation, the sum was 103.0 milliequivalents per liter, the increase being accounted for entirely by sodium. The calcium and magnesium values were consistent in all of the specimens analyzed.

As with other mammals, the changes in the total base concentrations as the animals approached the end of lactation indicate that as the offspring grows, the milk is modified in composition. This waning of lactation was marked by a diminution in yield and in content of solid matter. This milk with the lowered lactose and the increased salt content may well have been less appetizing to the suckling, apparently for the purpose of inducing the offspring to turn to other foods for sustenance. Also, it may be an adaptive mechanism, the nature of which is not yet understood. Since the mineral composition of milk "runs parallel to the stage of development of the embryo when born" ((14) p. 163), the total base concentrations of the milks of those mammals of comparable maturity at birth are similar.

#### SUMMARY

Milks obtained from three living bottle-nose dolphins and from a spotted dolphin 1.5 hours after death have been analyzed for their major chemical constituents. Dolphin milk, compared with that of most species, was found to be high in fat (108 to 180 gm. per liter) and protein (94.2 to 111 gm. per liter), and low in lactose (3.9 to 7.7 gm. per liter).

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# A TEST OF 2-KETO-L-GULONIC ACID FOR ANTI-SCORBUTIC PROPERTIES

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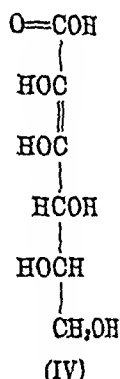
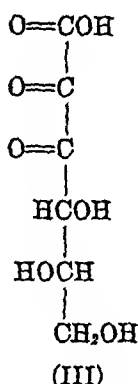
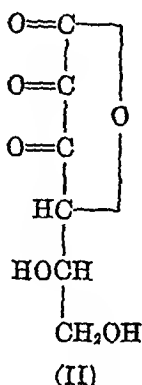
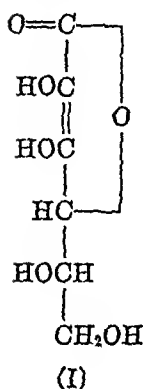
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When ascorbic acid (I) undergoes oxidation in a neutral medium, the oxidized product (II) first formed undergoes a fairly rapid transformation into more acid substances. As discussed previously (1), this change would appear to involve first an opening of the lactone ring to yield 2,3-diketo-l-gulonic acid (III). Further changes may then occur. Regeneration of ascorbic acid from such transformation products cannot be accomplished by the same simple reduction procedures that are effective for the conversion of dehydroascorbic acid (II). Now dehydroascorbic acid, which is stable in mildly acid solutions, has been shown to possess antiscorbutic properties (2, 4). The question therefore arises whether the animal body is capable of reconstituting ascorbic acid from 2,3-diketo-l-gulonic acid (III). If not, it would appear that opening of the lactone ring is equivalent to destruction of the vitamin and that when the vitamin is oxidized in the body it must be rapidly reduced again if its destruction is to be prevented. Borsook *et al.* (2) have reported that ascorbic acid oxidized *in vitro* in neutral or alkaline solutions and allowed to undergo the transformation discussed above possesses no antiscorbutic potency. They have therefore concluded that diketogulonic acid does not have antiscorbutic properties. It appeared desirable, however, to test this premise further with material of known composition. The use of 2,3-diketo-l-gulonic acid for this purpose is not as suitable as the more stable reductant of this compound, 2-keto-l-gulonic acid or its dienol form (IV) which can be easily prepared in pure crystalline condition. Therefore since the first step in the conversion of 2,3-diketo-l-gulonic acid into



ascorbic acid by the tissues would appear to be its reduction to 2-keto-*l*-gulonic acid, a test of its antiscorbutic properties seemed worth while. Though this acid is an intermediate in the chemical synthesis of vitamin C, no test of its antiscorbutic properties appears to have been previously recorded. Such a test is here-with described.



### *Preparation and Properties of 2-Keto-*l*-Gulonic Acid*

Though ascorbic acid may be produced from 2-keto-*l*-gulonic acid by the removal of water to form a lactone ring, the reverse process does not occur readily. Ascorbic acid heated in 0.1 N sodium hydroxide at 100° for 2 hours in an atmosphere of nitrogen showed no loss in its reducing power towards 2,6-dichlorophenol indophenol. The open chain acid does not reduce this dye. The 2-keto-*l*-gulonic acid employed was, therefore, synthesized from *l*-sorbose<sup>1</sup> according to the method of Reichstein and Grüssner (5). The crystalline product obtained was recrystallized once from water. It melted with decomposition between 155-160°. Reichstein and Grüssner state that decomposition occurs at 171° but that the decomposition point varies with the mode of heating. When titrated with alkali, the acid required 94 per cent of the theoretical amount calculated for  $\text{C}_6\text{H}_{10}\text{O}_7$ , or approximately 100 per cent of the theoretical if 1 molecule of water of crystallization is assumed. A 0.025 M solution of the half-neutralized acid (total ionic strength = 0.025) had a pH value of  $2.81 \pm 0.02$  at 30°. Ascorbic acid under similar conditions possesses a pK' value of 4.13 (1). A solution containing 20

<sup>1</sup> The *l*-sorbose was kindly furnished by Chas. Pfizer and Company, Inc.

mg. of 2-keto-l-gulonic acid showed reducing action towards 2,6-dichlorophenol indophenol that was less than that produced by 0.05 mg. of ascorbic acid. When such a solution was heated for 2 hours at 100° in the absence of air, it acquired pronounced reducing properties towards the dye, which quantitatively corresponded to 13.7 per cent conversion to ascorbic acid. Under similar conditions Reichstein and Grüssner (5) report 14 per cent conversion of 2-keto-l-gulonic acid to ascorbic acid.

### *Feeding Experiments*

The antiscorbutic activity of the 2-keto-l-gulonic acid was tested by feeding it to guinea pigs. Male animals, all weighing about 200 gm., were fed *ad libitum* the basal diet of Sherman, La Mer, and Campbell (6) with bran added as suggested by Eddy (3). Five sets of two animals each were employed and received the following additional supplements which were given daily by direct oral administration.

*No Additional Supplement*—The two animals in this set both died on the 27th day after they were placed on the experimental diet. Both animals lost weight rapidly during the last week and on autopsy showed the characteristic manifestations of scurvy: beading and enlargement of the costochondral junctions, marked hemorrhages throughout the body, fragility of the bones, looseness of the incisors, and enlargement of the knee joints.

*Ascorbic Acid, 2 Mg. Daily*—The two animals in this set showed moderate weight gain and were sacrificed on the 27th and 33rd days of the experiment. Both animals on autopsy appeared perfectly normal.

*2-Keto-l-Gulonic Acid, 2 Mg. Daily*—One of the animals in this set lost weight rapidly and died on the 22nd day of the experiment. The leg joints showed some swelling and the epiphyses were suspiciously weak, but other signs of scurvy were not evident on autopsy. The other animal also lost weight rapidly but did not die until the 32nd day. Here the characteristic symptoms of scurvy had more time to develop and were as severe as those found in the animals on the basal diet alone.

*2-Keto-l-Gulonic Acid, 5 Mg. Daily*—One animal in this set died after rapidly losing weight on the 17th day. On autopsy no indications of scurvy were found. The other animal died on the

24th day and showed on autopsy manifestations of scurvy which, though not as marked as in the case of the animals on the basal diet alone, were unmistakable.

*2-Keto-*l*-Gulonic Acid, 12 Mg. Daily*—One animal in this set died on the 14th day. Autopsy revealed pneumonia as the cause of death. The other animal was sacrificed on the 20th day and showed unmistakable signs of mild scurvy on autopsy.

#### SUMMARY

The results of the feeding experiments are not conclusive enough to state that 2-keto-*l*-gulonic acid possesses no antiscorbutic properties. There is an indication that at the higher dosage levels of this acid the scurvy symptoms were less severe but it must be remembered also that at these higher dosage levels the small percentage (0.1 to 0.2 per cent) of ascorbic acid present as an impurity in the preparation begins to reach amounts that are biologically effective. The experiments do indicate, however, that this acid is far less effective in preventing scurvy than is ascorbic acid when fed in similar amounts to guinea pigs. It would, therefore, appear that no appreciable regeneration of ascorbic acid in this animal occurs from 2, 3-diketo-*l*-gulonic acid by way of 2-keto-*l*-gulonic acid. This suggests that the opening of the lactone ring in vitamin C is tantamount to loss of antiscorbutic properties. The simultaneous loss of reducing and antiscorbutic properties on opening of the lactone ring further suggests that the reducing action of ascorbic acid is in some way associated with its biological function.

I am indebted to Dr. Barnett Cohen for his expert advice and assistance in diagnosing the scurvy symptoms.

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# THE GROWTH METABOLISM OF RHIZOBIUM, WITH EVIDENCE ON THE INTERRELATIONS BETWEEN RESPIRATION AND SYNTHESIS

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Numerous cultural studies with rhizobia, reported during the past 50 years, have established that these organisms have a comparatively simple metabolism. The quantity of sugars consumed in respiration in comparison with that consumed in growth is smaller than for most bacterial species. The formation of intermediate products of carbohydrate oxidation, such as organic acids and alcohols, is usually almost negligible. The main by-product ordinarily formed is a complex gum (7), the quantity varying markedly with the bacterial strain and growth conditions. This paper presents manometric and analytical data dealing with the respiration and growth metabolism of a strain of *Rhizobium* grown under conditions such that practically no by-products were formed. The carbohydrate consumed is accounted for quantitatively and its efficiency of utilization calculated. From these data some idea can be had of the interrelations existing between respiration and growth.

## Methods

The general procedure was to culture the organisms in a thin layer in large Erlenmeyer flasks and aseptically to remove 4 to 10 cc. samples at various times, starting as soon as there was sufficient growth to study, and continuing until growth stopped. The samples were examined for oxygen consumption, CO<sub>2</sub> production, respiratory quotient, dry weight of organisms, composition of organisms, composition of medium, and efficiency of growth. Analyses were made directly on the growing cultures,

and not on "resting cells" centrifuged from the medium. The technique used was worked out in an effort to understand both the resting oxidation and growth metabolism, especially the use which the organism makes of the carbohydrate.

*Organism Studied*—*Rhizobium meliloti*, No. 131, of the University of Wisconsin cultures, obtained through the courtesy of Dr. I. L. Baldwin, was used. This strain produces a negligible quantity of gum on our medium, is a rapidly growing organism of high infectiveness and effectiveness, and has been used in much of our previous work.

*Culture Conditions*—The organisms were grown on a sucrose-inorganic salts medium (1) containing iron and coenzyme R (6), with ammonia or nitrate nitrogen added as desired. The bacteria were grown at 28° in 2 liter Erlenmeyer flasks containing medium to a depth of about 1 cm. The initial pH was 6.8 but as growth proceeded the ammonia cultures tended to become slightly more acid and the nitrate ones more alkaline. The cultures were not aerated but were shaken well each day and before sampling.

*Respiration Measurements*—The usual Warburg technique (3) was used at 28°, the measurements being made in duplicate. The respiratory quotient was determined by the first method of Warburg, in which the CO<sub>2</sub> is absorbed in Ba(OH)<sub>2</sub> as the O<sub>2</sub> consumption is measured, and subsequently displaced by HCl tipped in from the side arm.

*Growth Measurements*—The quantity of organisms present in a sample was determined by centrifuging 10 cc. aliquots, washing once with distilled water, and determining oxidizable material by the method of von Fellenberg (4). The sample is digested in a mixture of 0.1 N K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> and concentrated H<sub>2</sub>SO<sub>4</sub> and diluted, KI is added, and the iodine, set free by the excess chromic acid, titrated with 0.1 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Digestion for 1 hour on a steam bath gave better reproducibility than 15 minutes at room temperature as in the original method. An empirical factor of 1.25 cc. of 0.1 N K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> per mg. of organisms was obtained by titration versus dry weight analysis.

*Determination of Sugar and Extracellular Growth Products*—The modified procedure of von Fellenberg as given above was also used for the determination of oxidizable materials left in the medium

after centrifuging. The accuracy of the method for determination of sucrose with the theoretical factor was checked against optical activity determinations.

*Analysis of Dried Bacteria*—A sample of culture large enough to yield 10 to 20 mg. of dried bacteria was centrifuged, distilled water added and again centrifuged, and the bacteria finally dried in a vacuum desiccator over  $P_2O_5$ . They were analyzed for carbon and hydrogen by combustion and for nitrogen by the micro-Kjeldahl procedure. This latter method was checked against a micro-Dumas determination with excellent agreement.

TABLE I

*Analysis of Bacterial Culture Medium for Cell By-Products and Unused Sugar*

Sample	Sugar by titration	Average	Sugar by optical activity
	mg. per cc.	mg. per cc.	mg. per cc.
0.400 % solution of glucose	4.00 3.95 4.08	4.01	$3.79 \pm 0.20$
Centrifugate from 10 day culture grown on sucrose	13.52 13.14 13.45 13.29		
Centrifugate from 15 day culture grown on sucrose	6.60 6.66 6.68	6.65	$6.69 \pm 0.15$
Centrifugate from 30 day culture grown on sucrose	14.10 14.10		
		14.10	$13.15 \pm 0.15$

### Results

*Analysis of Medium for By-Products and Unused Sugar*—In Table I are reported analyses of the centrifugates from three typical cultures grown on sucrose, as well as of a standard glucose solution. The results obtained by titration are compared with those obtained by the optical activity method.

The analyses show no gum or other oxidizable by-products in the centrifugate from 10 and 15 day cultures. The only oxidizable material present was unused sugar, as evidenced by the close agreement between values obtained by the optical activity and von Fellenberg methods. Gum formation would be easily de-

tected, since according to Grieg-Smith (5) its specific rotation is  $29^\circ$ ; the value for sucrose is  $66.5^\circ$ .

The figures for the 30 day culture indicate that some 10 per cent of the extracellular material was not sucrose, but this fact is unimportant in these studies of growing bacteria, since 10 and 15 day cultures had already stopped growing without significant gum production. Undoubtedly a high percentage of the bacteria in the 30 day culture was dead and had undergone some autolysis.

TABLE II  
*Chemical Composition of Rhizobium meliloti*

Experiment No.	Nitrogen source	Age of culture	Carbon	Hydrogen	Nitrogen	Ash*
		days	per cent	per cent	per cent	per cent
I-7	KNO <sub>3</sub>	3	47.80	6.87	5.73 5.76	(5.00)
H-1	"	6	50.52	7.14	6.00 6.06	5.50
H-1	"	20	49.49	6.94	6.60 6.60	4.91
I-10	NH <sub>4</sub> Cl	4	50.20	7.19	6.15 6.24	(5.00)
H-2	"	20	49.47	7.07	6.27	4.79
Mean.....			49.50	7.04	6.17	5.07
" ash-free basis.....			52.14	7.42	6.50	0.00

The limits of all analyses are C 47.5 to 50.6, H 6.80 to 7.20, N 4.96 to 6.60.

\* The ash content of approximately 5 per cent is not to be considered as contamination with mineral salts. The medium before inoculation was clear except in the cultures of Experiments I-7 and I-10, where a turbidity due to insoluble phosphates was present and a large black ash (9 per cent) resulted on ignition. The analytical results on these two cultures are calculated to 5 per cent and the subsequent calculations of economic coefficient are also based on this ash content.

*Composition of Bacteria*—It was necessary to determine the empirical composition of the bacteria in order to know the type of synthesis occurring, and to check the validity and constancy of the von Fellenberg procedure as a measure of dry weight. Table II gives analyses of bacteria taken from cultures used in obtaining the respiration data plotted in Figs. 1 and 2.

The bacteria were remarkably constant in composition over the

entire growth period when grown on both nitrate and ammonia nitrogen. A mean value of the analyses (Table II), calculated on an ash-free basis, gives  $C_{2.4}H_{1.6}NO_{4.6}$  for the elementary composition, which was taken as  $C_{1.9}H_{2.2}N_2O_9$ . The sulfur content of

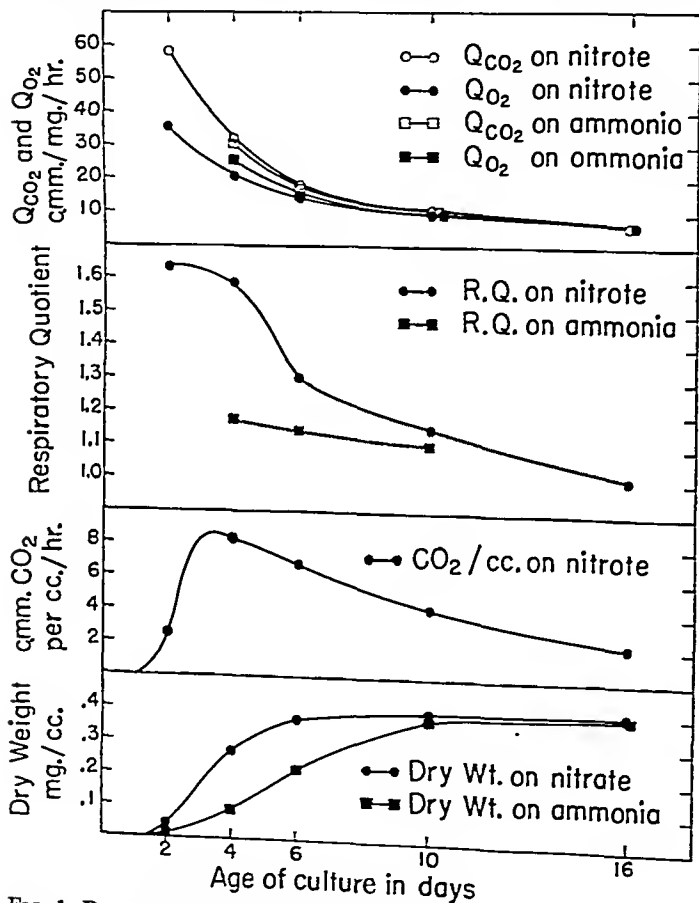


FIG. 1. Results of respiration measurements on cultures of *Rhizobium* grown on nitrate and ammonia nitrogen during a 16 day growth period.

the bacteria is not considered but this introduces only a minor error.

**Respiration Studies**—The respiration studies involved the determination of  $Q_{O_2}$  (cmm. of  $O_2$  per mg. of dry weight per hour),



$Q_{CO_2}$  (c.mm. of  $CO_2$  per mg. of dry weight per hour), and by calculation the R.Q. ( $Q_{CO_2}/Q_{O_2}$ ). The results of the measurements, usually continued over a period of 8 to 10 hours, are plotted in Figs. 1 and 2.

High values for  $Q_{O_2}$ ,  $Q_{CO_2}$ , and R.Q. were found in young cultures and all three decreased as the amount of growth approached the maximum. The  $Q_{O_2}$  values were lower and the R.Q. values much higher for nitrate cultures than for ammonia cultures, indicating that the oxygen of the nitrate was utilized. The final R.Q. value with both nitrogen sources approximated theoretic-

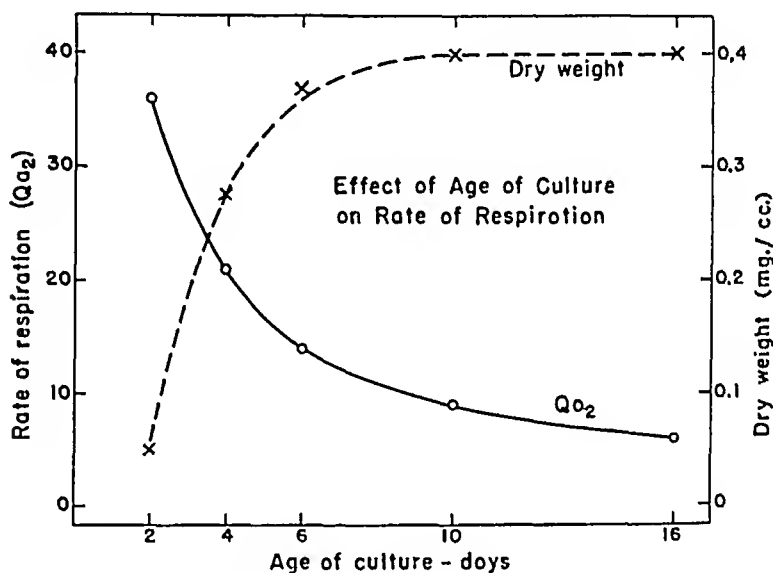


FIG. 2. Correlation between rate of growth and rate of respiration of *Rhizobium* grown on nitrate nitrogen.

cal for complete oxidation of sucrose and analysis showed a constant dry weight of organisms in the old cultures.

Interpretation of the respiration data is greatly facilitated by plotting the  $Q_{CO_2}$  and  $Q_{O_2}$  figures against each other, as in Fig. 3. This shows a linear relationship above approximately 8, which is the basal  $Q_{O_2}$  (or  $Q_{CO_2}$ ) for old resting cultures, as well as for young centrifuged and washed organisms that are truly resting (2). The slope of this line ( $Q_{CO_2}/Q_{O_2}$  - intercept) is interpreted as the R.Q. of growth, since growth and normal complete oxidation of

sucrose with simultaneous reduction of nitrate where present have been shown to be the only measurable reactions occurring. The growth R.Q. thus determined has a value of 1.86 for the nitrate cultures and 1.20 for those supplied ammonia nitrogen.

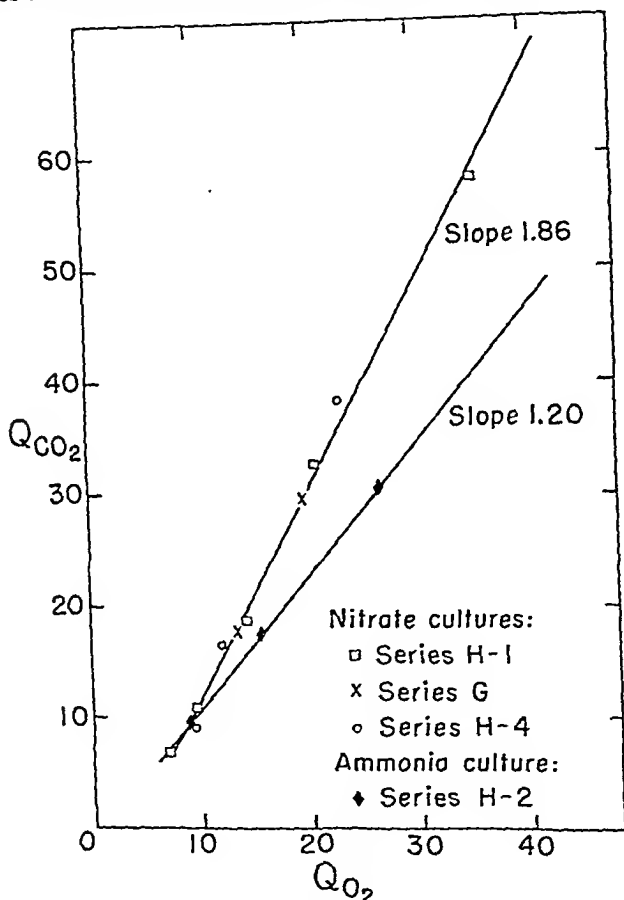
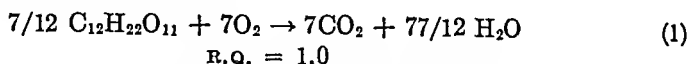


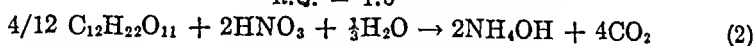
FIG. 3. A plot of  $Q_{CO_2}$  versus  $Q_{O_2}$  values for cultures of *Rhizobium* grown on nitrate and ammonia nitrogen.

An over-all equation of growth, based upon the separate reactions mentioned, can be set up to fit the growth R.Q., assuming that sucrose and nitrate are utilized to produce organisms of the

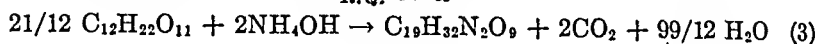
composition previously determined (Table II). These reactions may be represented as follows:



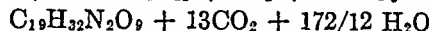
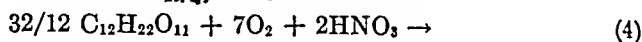
$$\text{R.Q.} = 1.0$$



$$\text{R.Q.} = \infty$$

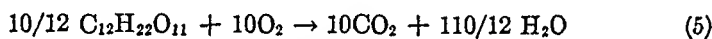


$$\text{R.Q.} = \infty$$

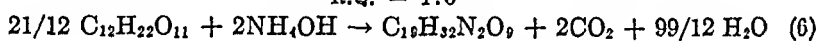


$$\text{R.Q.} = 1.86$$

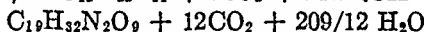
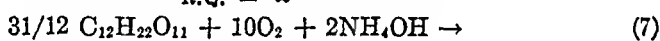
Similar equations can be written to fit the growth R.Q. of 1.20 observed with ammonia nitrogen, since these organisms had the same composition as those grown on nitrate. These are as follows:



$$\text{R.Q.} = 1.0$$

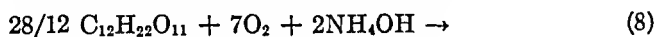


$$\text{R.Q.} = \infty$$



$$\text{R.Q.} = 1.20$$

These equations indicate that about 3 per cent less sucrose was consumed in producing a given quantity of bacteria with ammonia than with nitrate nitrogen. This is in general agreement with expectations, since nitrate reduction (Equation 2) requires sugar. If no more energy were required for Equation 6 than is represented by Equation 1 the over-all growth equation would be



$$\text{R.Q.} = 1.29$$

There is little reason, however, to expect synthesis according to Equation 8, since over half of the sugar in Equation 2 is not used in nitrate reduction but is presumably available for growth.

The accuracy of the R.Q. value of 1.20 observed with ammonia nitrogen is less than that of 1.86 observed with nitrate, owing to the less steep slope (Fig. 3) and to fewer observations. The efficiency data given below and our knowledge of the energy requirements for nitrate reduction would indicate that the value of 1.20 is slightly low.

It is not to be inferred that the reactions take place as written. For instance, nitrate reduction probably never actually proceeds to  $\text{NH}_4\text{OH}$  but some such type of reaction does occur. Likewise, growth is of course far more complicated than the simple over-all reactions might indicate.

*Efficiency of Sugar Utilization*—The efficiency of carbohydrate utilization is often expressed as the economic coefficient (mg. of dry weight of organisms produced per 100 mg. of sugar consumed). Dry weight, if related to the equations given previously, must include the constituent ash (cf. foot-note to Table II) which is a part of the synthesized cell. Calculated on this basis and with no allowance for basal respiration a theoretical efficiency of 49.8 for Equation 4, 51.4 for Equation 7, and 56.9 for Equation 8 is

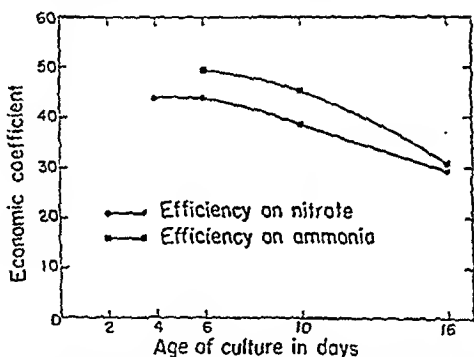


FIG. 4. Economic coefficients of cultures of *Rhizobium* grown on nitrate and ammonia nitrogen in the presence of excess sucrose.

obtained for the synthesis. The observed economic coefficient should approach nearest to these limiting figures in the youngest cultures. The values observed according to two methods are given below.

In the first method the economic coefficient was measured directly on aliquots of the cultures used in the respiration studies by determining sugar utilization per unit of dry weight synthesized. In these cultures a considerable excess of sugar was always present and the determinations were thus subject to rather large errors, since both growth and decrease in sugar concentration, especially in the young cultures, were small. The economic coefficients shown in Fig. 4 represent over-all values for the entire growth period. In general these values, as well as those obtained with

other cultures, show initial efficiencies of about 45, decreasing to 35 or 40 as growth reaches the maximum, and then falling still further as the rate of growth becomes negligible. The values for young cultures are near theoretical and show that basal metabolism consumed only a small portion of the sugar. The efficiency was higher for organisms grown with ammonia than with nitrate nitrogen, in agreement with theory.

In the second type of determination of efficiency, growth was limited by the amount of sucrose present and any significant production of chemically oxidizable by-products would have been evidenced both by direct determination and by a lesser amount of synthesis. The period before measurable growth occurred

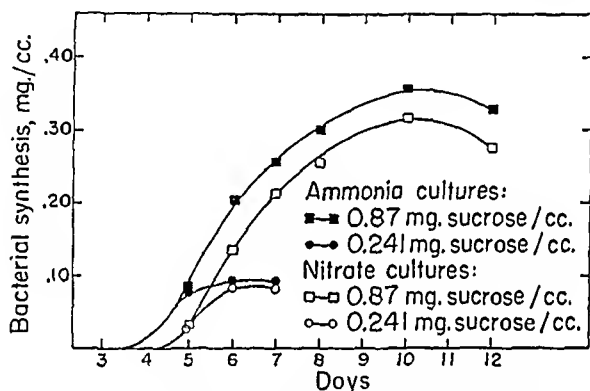


FIG. 5. Economic coefficients of cultures of *Rhizobium* grown on nitrate and ammonia nitrogen in the presence of limiting amounts of sucrose.

was rather long because of the small inoculum used. The dry weights of bacteria (Fig. 5) show that during a 10 day period an 0.087 per cent solution of sucrose was *completely utilized* with an economic coefficient of about 40 with ammonia and 37 with nitrate. This was only 10 to 20 per cent less than the maximum amount of growth attained with excess sugar and very young organisms. These growth measurements are consistent with the respiration data (Fig. 3) and the results in Fig. 4. Incidentally, these efficiencies are much higher than those commonly reported for other species of bacteria.

#### DISCUSSION

The experimental observations and calculations made furnish considerable definite information on the interrelations existing

between growth and respiration. When the  $Q_{O_2}$  and  $Q_{CO_2}$  values of cultures at various stages of growth were plotted against each other (Fig. 3) a linear relationship was found to exist between values above the basal metabolism. The fact that the relationship is linear, and no products besides bacteria and  $CO_2$  were produced, furnishes very strong evidence that in a growing culture two types of reactions are involved, one is the simple respiration (basal metabolism) reaction and the other is the over-all growth reaction for which equations were established. Approximately the same quantity of sugar per unit weight of bacteria is consumed in basal metabolism for resting and for growing cultures. This follows from the linearity of the  $Q_{O_2}$ - $Q_{CO_2}$  relationship as well as from the  $Q_{O_2}$  observed (2) for young resting cells. The growth reaction is merely added to the low basal oxidation that is always present. The rate of growth is therefore proportional to  $Q_{CO_2}$  above the resting  $Q_{CO_2}$  of about 8; that is, to  $(Q_{CO_2} - 8)$ . A  $Q_{CO_2}$  of 48, for example, indicates that about five-sixths of the sugar being consumed is going to synthesis at the time of measurement. The rate of growth is likewise proportional to  $(Q_{O_2} - 8)$  provided the nitrogen source used does not carry utilizable oxygen.

The evidence also indicates that the growth reaction is itself made up of at least two types of reactions. One of these (Equation 6) involves the conversion of sugar and nitrogen into bacteria, and the other (Equation 1 or 5) represents the oxidation of sugar to  $CO_2$  and  $H_2O$ . One-fourth of the sugar used in the over-all growth reactions shown in Equations 4 and 8 (nitrate reduction being neglected for the moment) is consumed according to Equation 1; the corresponding fraction for Equation 7 is approximately one-third. Apart from basal metabolism, then, a considerable quantity of sugar is required to synthesize bacterial cells.

These studies show that the over-all growth efficiency of a culture can be determined either directly or from the  $Q_{CO_2}$ -time curves, allowance being made for basal respiration. In the present experiments, however, direct comparisons of efficiencies so determined show general but not quantitative agreement. This is not unexpected considering that the values in one case are for unaerated cultures kept in Erlenmeyer flasks and in the other case are for cultures kept in well aerated Warburg vessels during the period of the determinations.

Comparatively few studies have been reported in the past with microorganisms in which a complete accounting of the materials consumed and of the products formed was made, as has been done here with *Rhizobium meliloti*. These studies emphasize the need for additional investigations of this type.

#### SUMMARY

The results of respiration and chemical studies on growing cultures of *Rhizobium meliloti*, supplied with ammonia and nitrate nitrogen, are as follows:

1. Essentially the only products formed were  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , and bacterial cells of nearly constant composition with respect to age of culture and nitrogen source.

2. The  $Q_{\text{CO}_2}$ - $Q_{\text{O}_2}$  relationship was analyzed graphically, and empirical equations established that describe the basal respiration and synthetic growth reactions occurring.

3. The rate of growth was proportional to  $Q_{\text{CO}_2}$  above 8, that is to  $(Q_{\text{CO}_2} - 8)$ . It was also proportional to  $(Q_{\text{O}_2} - 8)$  if the nitrogen source used did not furnish available oxygen.

4. A theoretical maximum growth efficiency or economic coefficient was calculated from the above findings. Direct determinations of economic coefficient by two methods agreed well with the theoretical value. Values of 40 to 50 were observed for very young cultures.

The writers are greatly indebted to Mildred S. Sherman for the determinations of the chemical composition of the bacteria, and to C. A. Ludwig, D. Burk, and F. W. Minor for valuable criticisms.

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# A PERMANGANATE ACID ASHING MICROMETHOD FOR IODINE DETERMINATIONS

## I. VALUES IN BLOOD OF NORMAL SUBJECTS\*

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(Received for publication, March 7, 1940)

Although the importance of blood iodine determinations in the study of thyroid functions has been clearly established, different methods have given widely varying values (2, 3, 7-9, 12, 13, 19, 22, 24-26, 28, 31, 32, 35-38, 46, 50, 53). The present permanganate acid ashing method has been developed after discovery of some of the errors and difficulties in the older methods. Permanganate has previously been employed for the determination of iodine (17, 33)‡ but it has not been used in acid solution with substances such as blood, in which the amount of organic material is so large in proportion to the minute quantity of iodine.

10 cc. of oxalated blood are oxidized with permanganate, dilute sulfuric acid, and the catalysts, ceric sulfate and copper. Distillation of the iodine is effected with oxalic acid, which liberates free iodine from its oxidized compounds. The iodine in the distillate is oxidized to iodate by permanganate as recommended by Groák (15), and is then determined quantitatively by a starch-thiosulfate

\* Some of the data in this paper are taken from the thesis presented by D. S. Riggs in partial fulfilment of the requirements for the degree of Doctor of Medicine, Yale University, 1939.

† Theresa Seessel Fellow, Department of Physiology, Yale University School of Medicine, 1939-40.

‡ White and Rolf have recently published (54) a method for the determination of diodrast and inorganic iodide iodine in urine, plasma, and trichloroacetic acid filtrates of blood or blood cells. This is apparently a modification of Groák's method which is used in the final step of our procedure. White and Rolf's method is not applicable to the small amounts of iodine occurring in normal blood.



titration. The iodine in inorganic and organic compounds has been recovered in 59 analyses, in thirty-four of which the reagents were added directly to the compounds, and in twenty-five of which the reagents were added to a mixture of these compounds and blood. Duplicate determinations on twenty samples of normal blood have been made by this method.

The discrepancies in blood iodine values are undoubtedly caused by certain difficulties which were encountered when both the alkaline ashing procedure and the chromic acid distillation technique were tried in our laboratory.<sup>1</sup> In the alkaline ashing methods (7, 11, 12, 16, 20, 27, 31, 35, 45, 47, 53) the blood is heated with strong alkali to a fairly high temperature until destruction of the organic matter is complete. The charred mass is extracted with alcohol and the extract is evaporated to dryness. Iodine in the residue is determined by one of various methods. The volatility of many iodine compounds at high temperatures, the difficulty of complete ashing without high temperatures, the danger of losses from many transfers and incomplete extraction, and the difficulty of obtaining iodine-free alkaline reagents contribute to the error of these methods.

In the acid ashing methods (3, 10, 18, 23, 50, 52) blood is digested with chromic acid in sulfuric acid solution, thus oxidizing the iodine to iodate or iodine pentoxide. A suitable reducing reagent converts the iodine to the free form which can then be separated from the digest by distillation. This method has the advantages over the alkaline ashing method that it is quicker, permits complete oxidation of blood without the danger of volatilization of iodine compounds, and eliminates the transfers and difficulties of extraction. It has the disadvantages that in the older complicated apparatus vacuum or steam distillation is necessary to procure quantitative iodine recoveries, and that chromium compounds may contaminate the distillate and interfere in the final step of the procedure.

Some oxidative step just before titration is essential in both the alkaline and acid ashing methods. Oxidation by bromine or chlorine has been used extensively but even minute quantities of excess bromine or chlorine may interfere seriously in the final

<sup>1</sup> Durlacher, S. H., Blood iodine determination, unpublished thesis presented for the degree of Doctor of Medicine, Yale University, 1938.

titration. Matthews, Curtis, and Brode (23)<sup>2</sup> eliminated the use of bromine by adapting Groák's method of oxidation of the distilled iodine by permanganate. However, with chromic acid, volatilization of chromyl chloride or similar compounds may lead to high values. In our method any manganese compounds appearing in the distillate do not interfere, since they are completely reduced together with the excess permanganate used in the final step to oxidize the iodine. Thus the danger of contamination of the distillate is eliminated and a simplified distilling apparatus without entrainment traps can be used. The distilling apparatus is indeed so simple that iodine can be carried over quantitatively without steam or vacuum distillation.

### *Apparatus*

The all-Pyrex distilling apparatus<sup>3</sup> with interchangeable ground glass joints is drawn to scale in Fig. 1. It consists of the following parts: *A*, digestion flask made from a 900 cc. Kjeldahl flask of which the long neck has been replaced by a ground glass joint; *B*, distilling arm connecting the flask with the condenser by two inside ground glass joints. The capillary tube attached to the dropping funnel extends to within 8.0 mm. of the bottom of the flask, *A*. The cross arm from the center of the capillary to the center of the condenser tube is 135 mm. long; *C*, short coil condenser, the water jacket of which measures 170 mm. Below the water jacket is a dew cup; *D*, receiving flask made by cutting off the top of a thin glass, 250 cc. wide mouth Erlenmeyer flask. At the base is joined a short horizontal side arm with a capacity of approximately 3 cc. and calibrated at 2 cc.; *E*, the thermometer, 10 cm. long and calibrated in 1° divisions from 120–200°, is attached by a heavy rubber band to the capillary tube of the distilling arm.

Thermometer. 35 cm. long and graduated from 0–200°.

Antibumps. 17 and 13 cm. long. A satisfactory type is made

<sup>2</sup> Matthews, N. L., The determination of iodine in biological materials, unpublished thesis presented for the degree of Master of Science, Ohio State University, 1937.

<sup>3</sup> This apparatus has been made for us by the Macalaster-Bicknell Company, 412 Temple Street, New Haven, Connecticut.

from a short length of glass tubing joined end to end with a glass rod.

Fisher burners.

Electric hot-plate.

Sand bath. A Pyrex pie plate covered with a thin layer of iodine-free sand serves as a convenient water bath for the oxidation step.

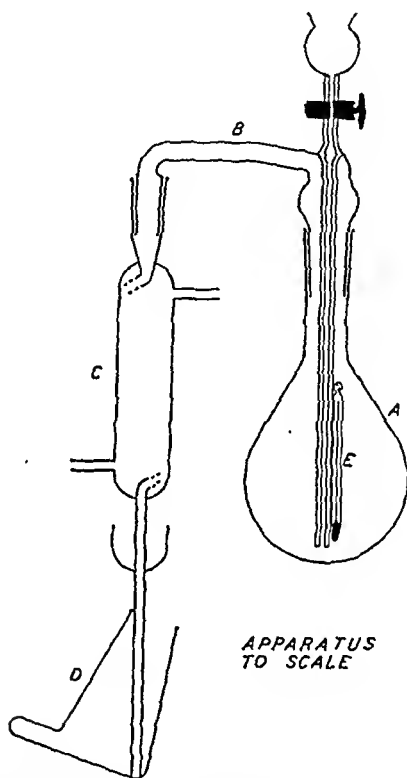


FIG. 1. Distilling apparatus. *A*, digestion flask; *B*, distilling arm; *C*, short coil condenser; *D*, receiving flask; *E*, thermometer.

Rehberg microburette similar in length and capacity to that described by Rehberg (41). The tip is attached to the burette by a ground glass joint and the terminal portion is drawn into a very fine capillary which dips under the surface of the solution during titration.

Wash bottle. The Corning washing bottle No. 1110 (1933 catalogue) is satisfactory but it should be provided with a saliva trap.

*Preparation and Purification of Reagents*

Where not otherwise specified, analytical reagent chemicals are used. Redistilled water is used not only in the preparation of solutions but also to rinse all apparatus.

Redistilled water. Approximately 200 gm. of potassium carbonate or hydroxide and a few glass antibumps are placed in a special 12 liter round bottomed distilling flask mounted on a shallow sand bath. The flask is half filled with distilled water, and connected to a water condenser by means of a ground glass joint. The sand bath is heated and the distillate is discarded until it becomes neutral to methyl red. More distilled water is added to the distilling flask as it is needed. The alkali should be replaced every 2 or 3 weeks.

0.1 per cent potassium oxalate solution (39).

Potassium permanganate. 700 gm. of crystalline potassium permanganate are dissolved as rapidly as possible by heating with 2500 cc. of water. The crystals should be stirred frequently until dissolved. As soon as the last crystals have disappeared, the solution is filtered through glass wool into a beaker and is stirred constantly while the beaker is chilled in ice water. The permanganate is suction-filtered on Whatman No. 42 filter paper in a Buchner funnel, washed five times with cooled redistilled water, transferred to a large porcelain evaporating dish, and dried in an oven overnight at 100°. It may be necessary to repeat this procedure once or twice.

1 per cent potassium permanganate solution is made from the recrystallized reagent.

0.2 M potassium permanganate solution. 31.6 gm. of recrystallized reagent are diluted to 1000 cc. with redistilled water. This solution should be stored in a brown bottle in the dark. If a precipitate forms the solution should be filtered through a glass fritted funnel.

Copper metal, granular.

Ceric sulfate.<sup>4</sup> 15 gm. of ceric sulfate are washed with 75 cc. of boiling 95 per cent redistilled ethyl alcohol. The ceric sulfate is allowed to settle and most of the supernatant alcohol is decanted. The remainder is filtered through a small filter paper.

<sup>4</sup> G. Frederick Smith Chemical Company; Columbus, Ohio.

This procedure is repeated twice and the ceric sulfate is then dried in air at room temperature and stored in the refrigerator.

18 N sulfuric acid (Merck or Mallinckrodt, c.p. analytical reagent, low nitrogen). Concentrated sulfuric acid is poured slowly into redistilled water in the proportion of 17 cc. of acid to 20 cc. of water to make 34 cc. of solution. The sulfuric acid has been found to be essentially iodine-free. Boiling the concentrated sulfuric acid for several hours did not alter the blank appreciably.

8 N sulfuric acid.

1 M potassium carbonate solution.

0.1 M sodium bisulfite solution.

Oxalic acid solution saturated at 30°. 500 gm. of oxalic acid (Mallinckrodt) are dissolved by heating to 90° in 400 cc. of water. The hot solution is filtered through fluted Whatman No. 1 filter paper into a beaker and is stirred constantly while the beaker is chilled in ice water. The crystals are filtered by suction on a Whatman No. 42 filter paper in a Buchner funnel and are washed five times with chilled water. The crystals are kept in the dark at room temperature in a brown bottle with sufficient water to make a saturated solution, saturation being insured by a residue of undissolved acid. Just before use the solution is heated to approximately 30°.

0.75 N sodium nitrite solution, 1.3 gm. per 50 cc. This solution should be made up at least every 2 weeks.

5 M urea solution.

1 per cent stabilized arrowroot starch solution. 10 gm. of arrowroot starch are rubbed up with a little water and poured, with stirring, into 1000 cc. of boiling water. The solution is removed from the flame at once and allowed to cool. 1 gm. of salicylic acid is added as a preservative. The solution will keep indefinitely in the refrigerator but should be centrifuged before use.

0.2 per cent potassium iodide solution. This must be freshly prepared daily.

0.001 N sodium thiosulfate solution. Approximately 26.5 gm. of sodium thiosulfate are diluted to 1000 cc. and the solution is allowed to stand for 2 weeks in order that sulfur may be precipitated. This stock solution (approximately 0.1 N) keeps

well if stored in the dark. The dilute solution, 1 part of stock solution made up with water to 100 cc., should be prepared not less frequently than once every 2 weeks and kept in the refrigerator. It is standardized as follows: 2 cc. of 0.01 N biiodate solution are pipetted into each of two titration flasks, 1 cc. of 1 per cent potassium iodide solution is added, and the flasks are cooled in ice water. 10 drops of 8 N sulfuric acid are added and the solution is immediately titrated with 0.001 N thiosulfate solution delivered from a 50 cc. burette calibrated at 0.1 cc. intervals. When the yellow color of free iodine has almost disappeared from the solution, 6 drops of 1 per cent starch solution are added and titration is continued to the disappearance of the blue starch-iodine color. The corrected titers of the two samples should check within 0.1 cc. The thiosulfate factor is obtained by dividing the theoretical titer (20.00 cc.) by the average of the observed titers. If the thiosulfate factor is too great, the concentration of the stock solution should be adjusted by appropriate dilution.

0.1 N biiodate solution (39).

### *Procedure*

*Digestion*—All determinations and blanks are carried out in duplicate. 14 gm. of recrystallized potassium permanganate, a small piece of copper, and approximately 10 mg. of ceric sulfate are mixed with 15 to 25 cc. of water in a digestion flask. While the mixture is shaken, 10 cc. of oxalated blood are added. 10 cc. of 18 N sulfuric acid are then introduced and the flask is again shaken vigorously. A violent foaming reaction takes place during which no more acid should be added lest the contents boil out of the flask. After approximately 5 minutes, when the reaction subsides, 200 cc. of 18 N sulfuric acid are added. Two large antibumps and a 200° thermometer are placed in the flask which is supported on an asbestos wire gauze, on a ring-stand. Heat from a Fisher burner is applied gently at first and the flask is shaken until boiling commences. Just before this point foaming occasionally is excessive and it may be necessary to stop heating momentarily and to add a little water from a wash bottle. As soon as the mixture is boiling steadily, no further shaking is necessary and the flame may be increased. Digestion is contin-

ued until a temperature of  $195^{\circ}$  is reached. The entire heating should take about 25 to 30 minutes.

When the digest has cooled to below  $100^{\circ}$ , 150 cc. of 1.0 per cent potassium permanganate solution are added. The flask is reheated to  $145^{\circ}$  during about 15 minutes. Constant shaking is necessary during this reheating in order to prevent bumping. When the digest has again cooled to below  $100^{\circ}$ , the thermometer is washed with 25 cc. of water and removed.

*Distillation*—The digestion flask is attached by means of the ground glass joint to the distilling apparatus. The receiving flask, containing 1.0 cc. each of 1.0 M potassium carbonate and 0.1 M sodium bisulfite, is tilted so that the tip of the condenser dips below the surface of the fluid. Heat is applied until the temperature of the digest reaches  $138$ – $140^{\circ}$ . Saturated oxalic acid solution is added through the dropping funnel slowly enough so that the rate of the evolution of carbon dioxide is moderate and the temperature does not fall below  $135^{\circ}$ . Complete reduction is indicated by decolorization of the digest, although a few minutes may be required for the last black specks to disappear. At least 2 or 3 cc. of oxalic acid in excess should be added to insure complete liberation of the iodine. A greater excess is inadvisable for the reason that will be mentioned under "Oxidation." Until the digest is completely reduced, continuous shaking is necessary to prevent bumping. While distillation is continued slowly, water is added from time to time through the dropping funnel to keep the temperature between  $135$ – $145^{\circ}$ . About 170 cc. of distillate should be collected during at least 30 minutes.

The receiving flask is provided with a small antibump. On an electric hot-plate or an asbestos mat over a Fisher burner the distillate is evaporated to approximately 20 cc. The antibump is washed down and removed and evaporation is continued more slowly to prevent spattering until the volume is 6 or 7 cc.

*Oxidation, Modified from Groák (15)*—The flask is placed in a shallow  $70$ – $80^{\circ}$  water bath on a hot-plate and 8 drops of 0.2 M potassium permanganate solution are added. If the 8 drops are decolorized, more permanganate is added until a permanent purple color is attained. If too large an excess of oxalic acid was added during distillation, more permanganate is required. After 4 minutes, 10 drops of 8 N sulfuric acid are added. Carbon dioxide

from the carbonate is evolved but the purple color should persist. After 4 more minutes the flask is removed from the water bath and 0.75 N sodium nitrite solution is added dropwise with constant shaking until the solution is water-clear and no specks of manganese dioxide remain. 1 drop of nitrite is added in excess and the sides of the flask are carefully washed by rotating the flask. 2 drops of 5 M urea are added at once and the sides of the flask are again carefully washed. The addition of the nitrite and urea should take about 1.5 minutes. The flask is replaced in the water bath until the solution comes to a volume of about 2.0 cc. Estimation of the volume is facilitated by the use of a special flask provided at the base with a short side arm calibrated at 2.0 cc. (see *D* in Fig. 1).

*Titration*—The flask is chilled in ice water and a drop of 1.0 per cent stabilized starch solution is added. 0.06 cc. of freshly prepared 0.2 per cent potassium iodide solution is pipetted into the solution and mixed by rotating the flask. Titration is effected with 0.001 N sodium thiosulfate solution delivered from a Rehberg microburette. When the blue starch-iodine color has almost disappeared, the flask is again chilled in ice water. As the end-point is approached, not more than 0.0005 to 0.0008 cc. of thiosulfate should be added at once. A comparison flask containing 2 cc. of water and a drop of starch facilitates accurate estimation of the end-point. During addition of thiosulfate the tip of the microburette should dip below the surface of the solution which is being titrated.

*Blank*—The technique for determining blanks is essentially the same as that used for blood except in two respects. The quantity of oxalic acid should be similar to that required in an analysis of blood. Therefore the original digestion must be prolonged to attain a degree of reduction of the permanganate equivalent to that which occurs with blood. Iodine must be added after evaporation of the distillate because the value of the blank is so small that accurate titration is impossible.

15.5 gm. of permanganate, about 20 cc. of water, a piece of copper, approximately 10 mg. of ceric sulfate, and 210 cc. of 18 N sulfuric acid are mixed in the manner described for blood. The same amount of anticoagulant as that used for one aliquot of blood should be included. The digest is heated to 195°, allowed



to cool below  $100^{\circ}$ , and after the addition of 100 cc. of water is reheated to  $195^{\circ}$ . 85 cc. of water are used to wash the thermometer. Distillation and evaporation of the distillate are carried out as in the blood method. Before oxidation of the 7 cc. of distillate with permanganate a small accurately measured quantity of biiodate solution is added to each flask. (0.5 cc. of 0.00005 N biiodate is convenient.) On the same day it is advisable to perform control titrations on the same amount of iodate added to a mixture of 1 cc. of 0.1 M sodium bisulfite and 1 cc. of 1.0 M potassium carbonate and carried through the final oxidation and titration processes.

The value of the blank is the difference between the titers of the control iodate samples and of the distillate to which iodate was added. It does not exceed 0.0050 cc. of 0.001 N thiosulfate if the reagents have been carefully repurified.

### *Discussion of Method*

The reactions in which iodates are formed by the action of permanganate on compounds of iodine have been studied by many investigators (4, 6, 14, 15, 29, 34, 40, 42, 48, 49). They have worked in alkaline, acid, and neutral solutions. Whether iodine oxides would be formed quantitatively in an acid permanganate solution without interreactions with the chlorides and bromides normally present in blood was a problem. However, Groák had shown that bromine is volatilized when sulfuric acid and permanganate are boiled with bromides (14). During an iodine determination, as outlined in the procedure, a strong odor of halogens may be noticed after the addition of the sulfuric acid to the mixture of permanganate, catalysts, and blood. That no iodine is volatilized at this point is demonstrated by the recovery experiments. It seems justifiable to assume that there is no contamination from chlorides and bromides, because our values for iodine are such minute fractions of the amounts of these halogens in normal blood. Furthermore the normal range of blood iodine determined by this permanganate method agrees with the low values of other investigators, as will be discussed later.

Leipert (18) suggested ceric sulfate as a catalyst in his original chromic acid ashing method and copper is widely used to catalyze

digestion in the Kjeldahl method. The results of a few analyses of blood with and without the catalysts did not differ significantly, but enough experiments have not been performed to prove whether the catalysts are essential for the determination of iodine in substances other than blood.

It is exceedingly important to use dilute (18 N) sulfuric acid. Concentrated (36 N) sulfuric acid must not be used, because the concentrated acid reacts with permanganate, even in aqueous solution, to liberate *dangerously explosive* purple fumes of highly oxidized manganese compounds (29).

The second digestion with 1 per cent permanganate solution may not be essential, because in several instances in which this heating was omitted the results were identical with those from samples of blood heated twice. However, some decomposition products of organic material are liberated during the second heating. Also, in the distillation process, reduction with oxalic acid solution proceeds more smoothly if the digest has been reheated with 1 per cent permanganate.

During distillation it is extremely important that reduction should occur at a temperature above  $135^{\circ}$  and that distillation after the initial addition of the oxalic acid be continued for at least 30 minutes. When these specifications were not meticulously followed recoveries of iodine were low. Tests for carbon monoxide in the gas liberated during distillation with oxalic acid were negative.

Since the accuracy of the starch-iodine titration is influenced by a great many factors, consistent results depend very largely on maintaining constant conditions of titration. Some of these factors merit brief discussion. The acidity of the solution must be maintained within fairly narrow limits (5, 8, 33, 35, 52). Too small a concentration of acid gives low titers, while a large excess gives slightly high values. An acidity of approximately 0.6 N in the solution to be titrated has been found satisfactory. The use of phosphoric acid as recommended by Groák (15) and Matthews and associates (23)<sup>2</sup> results in a slow development of the starch-iodine color and erroneously high titers. The amount of potassium iodide added before titration must be carefully controlled (1, 5, 10, 35, 43, 50). In the presence of the large amount of salts in the solution after the Groák oxidation, a large

excess of potassium iodide results in a purple or reddish violet starch-iodine color and an indefinite yellowish end-point. If, on the other hand, an insufficient amount of iodide is added, the iodide-iodate reaction does not go to completion and titers are low. For amounts of iodate iodine up to 2  $\gamma$ , 0.06 cc. of 0.2 per cent potassium iodide solution is satisfactory. It is important to cool the titration flasks in ice water before, and at intervals during, titration because the sensitivity of the starch indicator varies inversely with the temperature (44).<sup>2</sup> The source of light should be of constant strength and not bright enough to produce eye fatigue. A small Eastman x-ray illuminator with a sheet of thin white paper across the screen to soften the light has been extremely serviceable. The maximum depth of color is seen if the receiving flask is held at an angle so that the solution forms a shallow pool.

Even under the most favorable conditions the starch indicator is never sufficiently sensitive to produce a visible color with minute traces of iodine. Therefore a small amount of iodine will remain untitrated when the starch-iodine color disappears at the observed end-point. Since this amount varies directly with the volume of the solution, it may be necessary to add a small volume correction to the observed titer (1, 10, 16, 23, 30, 35, 43, 44). It is advisable for each investigator to determine the correction independently, by comparing observed and theoretical titers of known iodine solutions. In this laboratory a volume correction of about +0.02  $\gamma$  (equivalent to about 0.0010 cc. of 0.001 N thiosulfate) per cc. of final volume has been employed.

It seems scarcely necessary to stress the importance of avoiding sources of iodine contamination when such small quantities of iodine as are present in blood are being determined. Tincture of iodine should never be used in preparing the skin for venipuncture, nor should it be used by any of the laboratory workers. Extreme care should be exercised to keep hands and clothing free from iodine-containing solutions which might inadvertently be transferred to apparatus or reagents. All bottles of chemicals which might liberate free halogens should either be kept securely stoppered or removed from the laboratory.

The amounts of the reagents used must be varied in proportion to the quantity of blood to be analyzed. The following formulæ express the quantities required.

Gm. of  $\text{KMnO}_4 = 1.2$  (cc. of blood) + 2. (Decolorization during digestion indicates that the amount of permanganate was not sufficient and results in a low recovery of iodine.)

Cc. of 18  $\text{N}$   $\text{H}_2\text{SO}_4 = 13.3$  (total gm. of  $\text{KMnO}_4$  used). (It is especially important to use sufficient 18  $\text{N}$  sulfuric acid, since, if less than the indicated quantity is used, a white precipitate forms during distillation and causes bumping.)

Cc. of 1 per cent  $\text{KMnO}_4 =$  approximately 10 cc. per gm. of  $\text{KMnO}_4$  originally used but this amount must never be less than 100 cc.

Cc. of  $\text{H}_2\text{O}$  after the second heating  $\approx$  1 cc. per 8 cc. of 18  $\text{N}$   $\text{H}_2\text{SO}_4$ .

### Calculations

$$\frac{100}{\text{Cc. blood used}} \times \frac{T - Bl}{0.0473} = \gamma \text{ iodine per 100 cc. blood}$$

$T$  = observed titer corrected for burette calibration, thiosulfate factor, and volume correction.

$Bl$  = titer of blank corrected for burette calibration, thiosulfate factor, and volume correction.

0.0473 = cc. of 0.001  $\text{N}$  thiosulfate equivalent to 1  $\gamma$  of iodine.

### EXPERIMENTAL

The permanganate method has been tested by analyzing various iodine compounds added to the reagents with and without blood, as shown in Tables I and II. Although individual recoveries ranged from 79 to 118 per cent, the average percentage recoveries of two or more check determinations lay between 89 and 103 per cent. 90 per cent of the 59 recoveries reported lay between 88 and 105 per cent. The average for the whole group was 95 per cent. The average error of the permanganate method, as shown by these experiments, is 6.5 per cent. As may be seen from the summary in Table II, recoveries were as satisfactory when the iodine was organic in form as when potassium iodide was added. In many of these experiments the iodine was added to blood which was also separately analyzed for its iodine content. The recoveries thus obtained by difference were as adequate as when the iodine compounds were digested with the reagents

alone. Prolonging the digestion by holding the digest at a temperature of 180–195° for half an hour did not increase the

TABLE I

*Recoveries of Inorganic and Organic Iodine Analyzed Separately and after Mixing with Blood*

No. of analyses	Iodine added		Blood	Total iodine found	Iodine in blood alone	Recovery of added iodine	
	As	Amount					
		γ	cc.	γ	γ	γ	per cent
12	KI	0.25		0.24		0.24	96
12	"	0.50		0.48		0.48	96
3	"	0.50	10	0.76	0.28	0.48	96
3	"	1.00	10	1.25	0.30	0.95	95
3	Thyroxine	0.37	10	0.73	0.35	0.38	103
3	"	1.61	10	1.87	0.41	1.46	91
3	"	1.61	10	1.84	0.33	1.51	94
3	"	2.48	10	2.74	0.41	2.33	94
2	Iodo-horse-albumin*	2.11		2.04		2.04	97
2	"	3.04	10	3.13	0.33	2.80	92
2	"T" peptone*	2.70		2.43		2.43	90
2	" " *	3.89	8.8	3.77	0.29	3.48	89
2	"D" " *	1.79		1.67		1.67	93
3	" " *	1.61	10	1.80	0.31	1.49	93
4	"T" peptide*	0.71		0.73		0.73	103

\* These compounds were kindly supplied to us by Dr. W. T. Salter (21), Harvard Medical School.

TABLE II

*Average Recoveries*

	Without blood		With blood		Total	
	No. of analyses	Recovery	No. of analyses	Recovery	No. of analyses	Recovery
		per cent		per cent		per cent
Inorganic.....	24	96	6	96	30	96
Organic.....	10	97	19	94	29	95
Total.....	34	96	25	94	59	95

yield of iodine. It is highly unlikely that any iodine compounds appearing in blood would be more difficult to digest than thyroxine

or Lerman and Salter's (21) synthetic iodized protein and its split-products. Average recoveries were somewhat more satis-

TABLE III  
Normal Blood Iodine Values by Various Methods

	Investigator	Year	Subjects with normal thyroids			
			No. of cases	Maximum	Minimum	Average
				$\gamma$ per 100 cc.	$\gamma$ per 100 cc.	$\gamma$ per 100 cc.
Alkaline ashing	Veil and Sturm (53)	1925	36	20.0	10.0	12.8
	Orr and Leitch (31)	1929	16	10.0	4.0	6.0
	Fowweather (12)	1930	13	5.2	3.6	4.3
	Davis, Curtis, and Cole (7)	1934	28	16.2	8.5	11.9
	Perkin and Cattell (36)	1936	114	12.0*	1.5	6.8
	McCullagh and McCullagh (28)	1936		12.0	8.0	10.0
	Grauer and Saier (13)	1939	9	3.9	2.3	3.0
Closed combustion methods	Baumann and Metzger (2)	1932		10.0	8.6	9.6
	McClendon and Bratton (26)	1938		14.0	2.0	
Chromic acid ashing	Leipert (19)	1934	40	17	9	13
	Stevens (50)	1937	15	6.9	3.3	4.8
	Baumann and Metzger (3)	1937	20	4.4	2.3	3.0
	Fashena (9)	1938	79†	12.0	3.0	6.6
	Matthews, Curtis, and Brode (23)	1938				4.0
	Grauer and Saier (13)	1939	9	4.5	2.2	3.2
Permanganate ashing method	Riggs and Man	1940	20	3.7	2.5	3.1

\* In 1938 Perkin and Lahey say, "10 micrograms of iodine per hundred cubic centimeters of whole blood is considered to be the upper limit of normal" (37).

† Children from birth to 13 years old.

factory when the amount of iodine added did not exceed 2.0  $\gamma$ . With larger amounts of iodine it is probable that the small quantity of potassium iodide added before titration is not quite suffi-

cient to force the iodide-iodate reaction to completion (35). Although the percentage error remains relatively constant for varying amounts of iodine, the absolute error (expressed in  $\gamma$ ) is much less when the amount of iodine to be analyzed is small, as in 10 cc. of blood.

A summary of the duplicate determinations of the blood iodine of ten normal male and ten normal female subjects is presented at the end of Table III. The average difference between duplicates was less than 0.2  $\gamma$  per cent. The average blood iodine for the whole group was 3.1, with extremes of 2.5 and 3.7  $\gamma$  per cent. The averages for the males and females were identical, 3.1  $\gamma$  per cent. However, when the number of our normal subjects is increased and when the analyses are continued throughout the year (most of our determinations were made in the fall and winter months), it is probable that the range of our normal values will be somewhat increased.

#### DISCUSSION

This permanganate distillation procedure gives iodine values in blood which agree with the low quantities found by some previous investigators. These values are presented in Table III together with a small selection of the results obtained by various alkaline ashing methods. Fowweather (12), Grauer and Saier (13), Stevens (50), Baumann and Metzger (3), and Matthews, Curtis, and Brode (23) report average blood iodines from 3.0 to 4.8  $\gamma$  per cent, which agree closely with our average value of 3.1  $\gamma$  per cent. Our maximum blood iodine is the lowest in Table III. This may be related to the fact that our normal subjects have been exceptionally careful to avoid any contact with iodine before the blood was taken. Perkin *et al.* (38) have reported that two surgeons who of necessity were exposed to iodine had blood iodines over 10  $\gamma$  per cent.

Of the nine average values in Table III which far exceed our average seven were obtained by some alkaline ashing method. Baumann and Metzger (3) and Trevorrow (51) have discussed the errors which give high results in the alkaline ashing methods. They state that after alcoholic extraction of iodine some organic material or a polymer from the alcohol in contact with alkali

interferes in the final titration and reacts with the thiosulfate to give high titers. Furthermore, the alkaline reagents are apt to include iodine impurities. In fact Perkin (35, 36) employs a blank of 0.2  $\gamma$  of iodine. This is more than the minimum of 0.15  $\gamma$  which he found in a 10 cc. aliquot of normal blood. Whatever the sources of error are, it is significant that Baumann and Metzger (3) and Curtis and his associates (23) did not corroborate their high values obtained by alkaline ashing methods when they changed to acid ashing procedures. However, Grauer and Saier (13) found almost identical average low values, 3.0 and 3.2  $\gamma$  per cent, when they compared alkaline and acid ashing methods. Of the values determined by the chromic acid ashing procedures only those of Leipert (19) and Fashena (9) exceed those obtained by our permanganate method. Leipert's use of arsenious acid as a reducing agent has been thought to give a "variable positive error" (51, 52). It is difficult to compare our results on normal adults with those of Fashena who worked with children from birth to 13 years in age and who has experienced difficulties with iodine contamination.<sup>5</sup> When the published criticisms of the alkaline ashing and Leipert's methods are considered, credence must be given to the low values for blood iodine of Stevens (50), Baumann and Metzger (3), Matthews, Curtis, and Brode (23), and Grauer and Saier (13). The iodine content of blood as determined by the permanganate method agrees closely with these low results.

#### SUMMARY

A method is described for the determination of iodine in blood. The blood is ashed with permanganate and sulfuric acid and the iodine is distilled from the digest after reduction with oxalic acid. The iodine in the distillate is oxidized to iodate by permanganate, the excess permanganate is reduced, and the iodate is titrated with thiosulfate after the addition of potassium iodide.

In 59 experiments inorganic and organic iodine has been recovered with an average error of 6.5 per cent. The average recovery for all experiments was 95 per cent.

The average blood iodine of ten normal male and of ten normal

<sup>5</sup> Fashena, G. J., personal communication.



female adults was 3.1  $\gamma$  per cent. These results are compared with those of other investigators.

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## IDENTIFICATION OF THE RICE FACTOR

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For some time (1, 2) there have existed indications that polished rice contains a chick growth factor not present in any significant amounts in yeast. Recently (3), it has been shown with a more complete diet that polished rice has a growth-promoting effect which cannot be provided by levels of dried yeast up to 15 per cent of the ration.

It is our purpose to present results of further investigations of the "rice factor" which have led to the identification of its components in pure form, or at least in the form of pure active substitutes.

### *Methods and Results*

The basal diets used in this work have varied in minor details, since the work has proceeded simultaneously in both laboratories. Essentially, the diets have contained casein 30 per cent, or water-washed sardine meal 24 per cent, yeast 10 per cent, wheat germ oil or soy bean oil 3 per cent, adequate levels of all the mineral elements, fish oil to supply vitamins A and D, and glucose or starch and sucrose to make up the balance. Single comb white Leghorn chicks were used as test animals and kept in metal, electrically heated, battery brooders. Experimental periods extended for 2 weeks or more.

In tests with the sardine meal diet, it was noted that growth was markedly increased when the pure carbohydrates were replaced by polished rice. However, the addition of dried cartilage to the basal diet also accelerated growth, indicating that cartilage possessed a growth factor similar to that in polished rice.

In later experiments with the principal components of cartilage, it was observed that both chondroitin and gelatin increased the gains made, but only partially, and that glycine, the principal amino acid in gelatin, could serve as well as the gelatin, indicating that the other amino acids of gelatin played no considerable part in the growth effects noted. It was then found that gelatin or synthetic glycine in combination with chondroitin<sup>1</sup> could cause a rate of growth equal to that observed in chicks on a cartilage-supplemented diet or on a rice-supplemented diet. These experiments

TABLE I  
*Influence of Various Supplements to "Rice Factor" Basal Diet*

Supplement*	Fish meal basal diet			Casein basal diet		
	Level	No. of chicks	Basal gain made	Level	No. of chicks	Basal gain made
	per cent		per cent	per cent		per cent
None		15	100		6	100
Glycine	2.0	15	103	1.0	7	100
Chondroitin	2.5	15	112	3.0	7	100
Gelatin	5.0	15	109	3.0	7	103
Glycine +	2.0	15	129	1.0	8	159
Chondroitin	2.5			3.0		
Gelatin +	5.0	15	143	3.0	7	162
Chondroitin	2.5			3.0		
Cartilage, dried	5.0	15	145	6.0	7	156

\* All supplements were used in replacement of an equal quantity of carbohydrate.

were then confirmed with a basal diet containing casein instead of sardine meal. Similar results have been obtained repeatedly in both laboratories. Typical data are given in Table I.

Arginine failed to increase the growth of chicks fed the basal diets plus glycine and chondroitin. Evidently, these basal diets were not deficient in arginine. Chicks fed practical rearing diets concurrently have shown essentially the same rate of gain as those on the most favorably supplemented experimental diets.

<sup>1</sup> The growth-promoting effect of the chondroitin preparation was not altered by further purification according to the lead salt procedure described by Hawk and Bergeim (4).

## DISCUSSION

Our investigations have shown that the "rice factor" can be identified with, or at least replaced by, a mixture of glycine and chondroitin. Either of these added to the sardine meal basal ration, which undoubtedly contained small amounts of both, usually increased growth slightly but the greatest restoration of growth was produced by a combination of the two supplements. It is evident that each is a distinct growth factor for the chick. A casein basal diet, which apparently contains less of both glycine and chondroitin, permits little or no increase of growth when either is added alone, but when combinations or sources of both are added growth is markedly accelerated.

In respect to chondroitin, our results confirm the conclusions of Robinson, Gray, Chesley, and Crandall (5) that this substance is (or contains) a growth factor for the chick. In respect to glycine, our results indicate for the first time that this amino acid is required in the diet for optimum growth of the chick. Further experiments will be needed to decide on the degree of the essential nature of glycine for the chick. These results with glycine are striking because of the generally accepted view that glycine is not an essential amino acid for the rat (6).

Attempts to employ glycine at levels higher than 1 per cent have not produced better growth. Levels higher than 2 per cent have given some evidence of greater mortality and a detrimental effect on growth. This is possibly allied with the toxic effect of glycine at high levels described by Patton (7).

Hegsted, Oleson, Elvehjem, and Hart (8) have reported on a growth factor in cartilage and rice which was capable of increasing the growth of chicks. Their basal diet was composed of casein 18, dextrin 64, soy bean oil 5, brewers' yeast 5, liver extract 3, vitamin A and D concentrate, and minerals. In contrast to cartilage and rice, chondroitin, edestin, and arginine, fed separately, were without effect. The basal diet used by these workers was deficient in chondroitin, glycine, and probably arginine (9, 10); hence supplements which did not provide all of these factors failed to increase growth.

We wish to acknowledge donations of chondroitin and dried cartilage by The Wilson Laboratories, Chicago, through the kindness of Dr. David Klein.

## SUMMARY

Glycine is required in the diet for optimum growth of the chick. When adequate glycine is present in the diet, chondroitin has a growth-promoting action on the chick. A combination of glycine and chondroitin can replace the "rice factor."

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# VARIATIONS IN THE BLOOD CALCIUM AND PHOSPHORUS WITH THE AGE OF THE DOG\*

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In a larger experiment designed to compare the nutritive values of commercially available milks, calcium and inorganic phosphorus determinations on the dog blood were included as part of the routine examination.

A progressive fall in the level of inorganic phosphorus of the blood of the puppies during the period of rapid growth raised the question whether this was a normal trend or one aggravated by a nutritional deficiency. Surveys of the literature showed no data which would correlate the levels of inorganic phosphorus with the age of the dog, so the following experiment was designed to determine the natural trends and to compare them with those obtained in a moderate vitamin D deficiency.

## EXPERIMENTAL

Weanling fox-terrier pups were employed in the major part of the study. Occasional analyses were made on growing pups from Airedale, great Dane, bull-terrier, and mongrel strains for comparison. The rations employed were for the most part mineralized milk.<sup>1</sup> Raw, pasteurized, and evaporated milks were used but since no significant difference occurred between these groups, with regard to the calcium and phosphorus levels, no distinction will

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<sup>1</sup> The daily supply of minerals included 5 mg. of iron as the pyrophosphate, 1 mg. of copper as the sulfate, 1 mg. of manganese as the chloride.



be made between them in this report. The other ration employed was a commercial dog food which has given satisfactory growth and reproduction.

The animals were kept in a pen of galvanized iron wire, on a dry concrete floor which was covered with shavings. They were fed twice daily in earthen jars which were raised above the floor to prevent waste and contamination. Supplements were added to a small amount of milk for the morning ration. The evening supply was large enough to insure *ad libitum* consumption.

Calcium and phosphorus determinations were made on the trichloroacetic acid filtrate of blood as described by Elvehjem and

TABLE I

*Increase in Blood Inorganic Phosphorus of Dogs, Due to Acid Hydrolysis*

Time	Inorganic P	Increase	
hrs.	mg. per cent	mg. per cent	per cent
0.25	3.05		
2.0	3.17	0.12	3.9
3.0	3.21	0.16	5.2
5.0	3.28	0.23	7.6
6.0	3.35	0.30	9.8
7.0	3.40	0.35	11.5
24.0	3.63	0.58	19.0

Kline (1). The blood was drawn from the jugular vein and discharged directly into the trichloroacetic acid. The phosphorus determinations were made with an Evelyn photoelectric colorimeter.

Preliminary surveys of the analytical procedure indicated two factors which might introduce errors in the results—the diurnal variation, and the increase of inorganic phosphate in the filtrate due to acid hydrolysis.

Shortly after a dog consumed milk, the inorganic phosphorus was found to increase as much as 30 per cent; otherwise the diurnal variation was practically negligible. The animals were therefore bled approximately 5 to 6 hours after the morning feeding and in relatively the same order.

Table I shows typical data for the increase of inorganic phosphorus due to hydrolysis in the trichloroacetic acid. All of the

readings reported here were made within 3 hours after bleeding; hence the error due to hydrolysis is probably no greater than 5 per cent, and in most cases considerably less. Bleeding the animals in the same order would also serve to make these errors constant; thus the data would serve well for establishing the general trends due to the maturation of the animals.

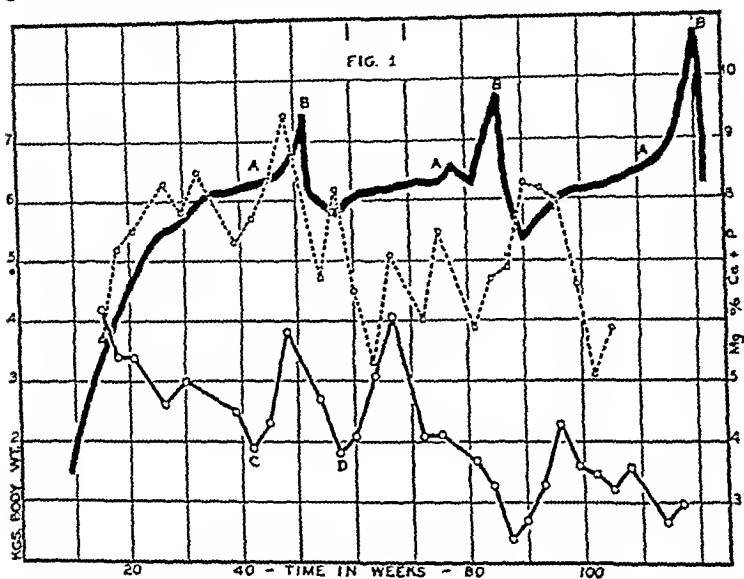


FIG. 1. Effect of vitamin D following vitamin D deficiency. The heavy curve = body weight, light curve = level of inorganic phosphorus in blood, dotted curve = level of calcium in blood. A = estrus, B = parturition, C = 30 U.S.P. units of vitamin D per day, D = 125 units of vitamin D per day.

In the attempt to produce a moderate vitamin D deficiency, five dogs (three females and two males) were placed on the mineralized milk rations with no additional source of vitamin D. After 42 weeks (Fig. 1) the low level of inorganic phosphorus suggested that they might be suffering from a moderate vitamin D deficiency. At this time the dogs were supplemented with 30 U.S.P. units of vitamin D per day as cod liver oil. The results with Dog 1 shown in Fig. 1 are quite typical of all the dogs. The rise in inorganic phosphorus of the blood of all five of the animals

following the administration of cod liver oil confirms the belief that a slight deficiency of vitamin D existed.

Following the initial response to the cod liver oil the level of inorganic phosphorus again decreased and now the level of the cod liver oil was increased to supply 125 U.S.P. units of vitamin D per day. Again a rise was obtained but again there was a rather rapid return to the original level, in spite of the continued administration of the cod liver oil.

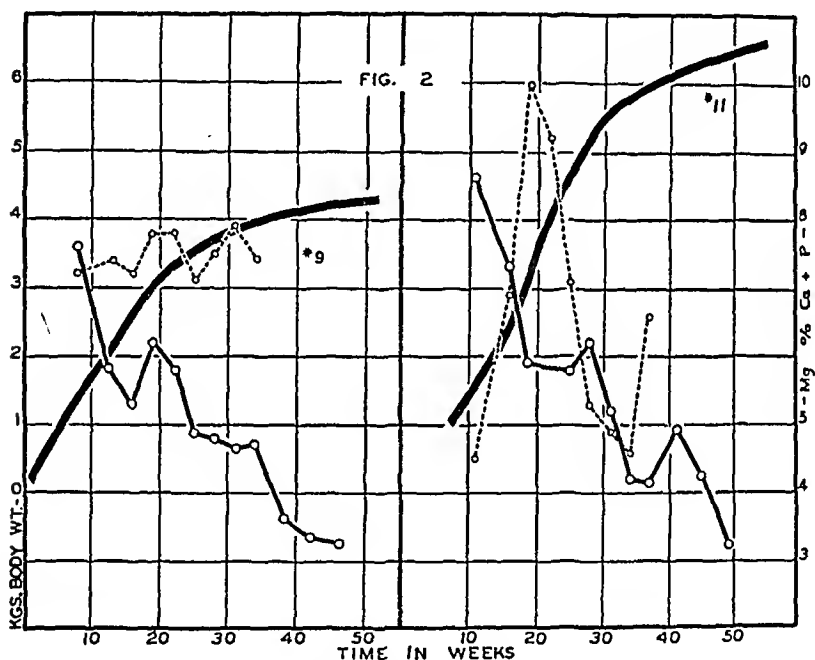


FIG. 2. Effect of vitamin D on dogs receiving adequate vitamin D since weaning. The heavy curve = body weight, light curve = level of inorganic phosphorus in blood, dotted curve = level of calcium in blood.

These results are typical of all five dogs of the original series. Each showed a temporary response which gradually subsided. In several cases, after the administration of cod liver oil, the inorganic phosphorus showed an initial drop, while the calcium rose sharply (in one instance reaching 14 mg. per 100 cc. of blood). These effects then gave way to the more general trends shown in Fig. 1. Although we have not ruled out the possibility of additional factors in the cod liver oil which might have influenced

the results, the majority of the results indicate that vitamin D was the primary factor concerned.

Fig. 2 presents data obtained from dogs receiving adequate vitamin D from weaning. Dog 9 received 125 U.S.P. units of vitamin D per day as cod liver oil in addition to mineralized milk. Dog 11 received the commercial dog food. Here again the general trend in the level of inorganic phosphorus was downward as the dogs developed.

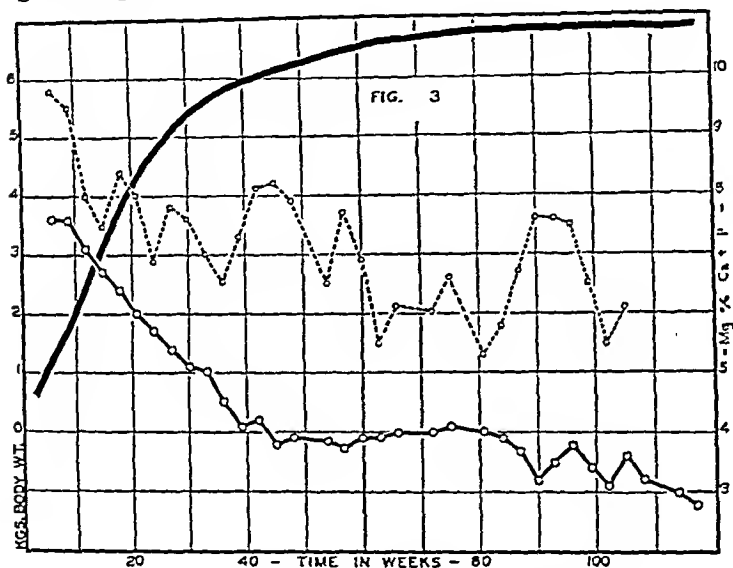


FIG. 3. Changes in blood of dogs from birth to 2 years of age. The heavy curve = body weight, light curve = level of inorganic phosphorus, dotted curve = level of calcium.

In Fig. 3 data obtained from approximately twenty dogs during the period of rapid growth are summarized; after the 1st year the data are based on only five dogs. The inorganic phosphorus decreases, following a rather smooth curve. Levels as low as 2.2 mg. per 100 cc. of blood have been observed, with no apparent ill effects to the dog. In general, however, the normal range for the mature dogs appears to lie between 2.6 and 4.0 mg. per 100 cc. of blood.

The calcium levels in all the animals were highly variable but

in general when a sharp drop occurred in the inorganic phosphorus, there was a simultaneous rise in calcium. Over the 2 year period, however, the general trend seemed to be downward.

Partition of the phosphorus of the blood from the original five dogs is summarized in Table II. The data are not complete enough to warrant definite conclusions but it would appear that the decrease with the age of the animal is not peculiar to the inorganic fraction but may take place in the other fractions as well.

The decrease in the inorganic phosphorus of the blood as the dogs become mature suggests a relationship between the actively metabolizing tissue and the level of blood phosphorus. The correlation does not exist to the same degree in the actively metabolizing tissues of pregnancy. Among the seven pregnancies so

TABLE II  
*Phosphorus Partition of Whole Blood in Dogs*

Age		Total P	Total acid-soluble P	Inorganic P
<i>wks.</i>		<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
80	High	49.5	33.3	4.8
	Low	38.4	15.4	2.2
	Average	45.1	24.4	3.9
120	High	40.0	21.8	3.4
	Low	38.5	18.0	2.5
	Average	39.2	19.7	2.8

far observed there was only a slight rise in inorganic phosphorus, which sometimes continued throughout the lactation period.

Since many of the existing data for inorganic phosphorus were based on analyses of serum, comparisons were made between the inorganic phosphorus of serum and whole blood. Results of thirty such determinations showed that a conversion factor of 1.4 would give an approximate value for both serum calcium and serum inorganic phosphorus when multiplied by the values obtained on whole blood.

#### DISCUSSION

From the data presented it is apparent that mature dogs have a distinctly lower level of inorganic phosphorus than do young growing dogs. Such a condition has been noted in sheep (2), and in cattle (3) especially during the suckling period. In non-suckling calves the levels appear to be only moderately higher

than in the adults. In dogs, however, we have shown this decrease to be a progressive one, extending well into the 2nd year of the animal's life.

Results of the phosphorus partition of the blood indicate that our data compare favorably with those of other investigators (4, 5). Since we obtained the same general trends on each of the rations employed, it appears that this is a normal condition.

Cheymol and Quinpuad (6) have reported a correlation between the calcium level of blood serum and the season of the year. Since the rises which they observed occurred in the spring and fall (at which time most of their dogs were in heat), they have suggested a correlation with the estrous cycle. Our ration is not comparable to the one they employed and therefore it is difficult to compare our data, but the rise from 10.8 to 11.3 mg. per 100 cc. of blood which they report as significant would be well within the weekly variations obtained with our animals.

#### SUMMARY

1. The inorganic phosphorus of dog blood was found to decrease progressively throughout the growing period.

2. A mild vitamin D deficiency was produced by feeding animals solely on mineralized milk. The administration of cod liver oil produced a rise in inorganic phosphorus and a fall in the level of calcium.

3. The level of blood calcium was found to be highly variable on the rations which we employed. The ultimate trend in the level of calcium during the period of maturation was downward.

4. Apparently the only correlation which could be made involving the calcium level was the sharp rise which occurred when there was a sharp fall in inorganic phosphorus.

5. Since we obtained similar results using several different rations, it appears that these are normal trends for the dog.

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# THE SPECIFICITY OF CARBOXYPEPTIDASE

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(Received for publication, March 18, 1940)

All the information hitherto available with respect to the specificity of carboxypeptidase has been obtained by the use of crude enzyme preparations (1). Consequently, there still remains some uncertainty as to whether the previously reported hydrolyses of various synthetic substrates, attributed to the action of carboxypeptidase, are all due to the same enzyme. Indeed, Abderhalden and Abderhalden (2) have advanced the hypothesis that the hydrolysis of chloroacetyltyrosine and of similar halogen-acylated amino acids and peptides is attributable to an acylase different from the genuine carboxypeptidase. The latter is assumed to attack only those polypeptides in which the amino group is masked by a benzoyl or naphthalenesulfonyl group.

The specificity of carboxypeptidase has now been reinvestigated by means of crystalline carboxypeptidase preparations obtained by the method of Anson (3). Some of the results of this investigation are reported in Table I. It will be noted that the amount of enzyme required for the hydrolysis of a substrate varies widely with the nature of the substrate.

The carbobenzoxyglycyl derivatives of several amino acids may be compared first. While carbobenzoxyglycylglycine is hydrolyzed rather slowly in the presence of 0.5 mg. of enzyme N per cc., carbobenzoxyglycyl-*l*-alanine requires only 0.08 mg. of enzyme N per cc. for a rather rapid hydrolysis. Finally, the carbobenzoxyglycine derivatives of *l*-phenylalanine and *l*-tyrosine are split in solutions containing only 0.3  $\gamma$  of enzyme N per cc.

The carbobenzoxyglycyl derivatives of *l*-phenylalanine and

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*l*-tyrosine are somewhat more sensitive toward carboxypeptidase than are the carbobenzoxy-*l*-glutamyl derivatives of these amino acids.

In order to reinvestigate the significance of the terminal carboxyl group, carbobenzoxy-*l*-glutamyl-*l*-phenylalanine and carbobenzoxy-*l*-glutamyl-*l*-phenylalanineamide were subjected to carboxypeptidase action. The relative amounts of enzyme employed for the experiments with the amide and the free acid were about 1000:1. Nevertheless, the amide was found to be resistant to enzymatic action, while the acid was hydrolyzed quite rapidly.

The typical substrates for carboxypeptidase do not contain any free amino group. It is therefore the more remarkable that  $\alpha$ -hippuryl- $\epsilon$ -carbobenzoxy-*l*-lysine is not split by carboxypeptidase in contrast to the sensitivity of  $\alpha$ -hippuryl-*l*-lysine. In this case, the peptide containing a free amino group is attacked by the enzyme. When the free amino group is masked, the peptide is rendered resistant toward the enzymatic action.

*l*-Tyrosyl-*l*-tyrosine and glycyl-*l*-tyrosine are other substrates for carboxypeptidase that contain free amino groups.

Benzoylglycylglycine is reported (1) to be resistant to the action of carboxypeptidase; however, it will be noted from Table I that when considerable amounts of the enzyme were employed, hydrolysis of this substrate could be effected. A still slower hydrolysis was found in the case of benzoyl-*l*-phenylalanine.

The fact that carbobenzoxyglycyl-*l*-glutamic acid is split rather slowly by carboxypeptidase seems also to be worthy of mention.

In the case of all the substrates hitherto subjected to the action of carboxypeptidase, the action of the enzyme has consisted in a splitting off of the amino acid residue bearing the terminal carboxyl group.

Chloroacetyl-*l*-tyrosine is generally regarded as the typical substrate for carboxypeptidase and is therefore frequently used to identify and to estimate this enzyme (10). The use of chloroacetyl-*l*-tyrosine for the estimation of carboxypeptidase is accompanied by two disadvantages. During the enzymatic hydrolysis free tyrosine crystallizes out, thus making the performance of a quantitative determination a difficult procedure. Of greater

TABLE I

*Behavior of Synthetic Substrates toward Crystalline Carboxypeptidase*

Substrate	Carboxy- peptidase, 3 times crystallized	pH	Time	Hydrolysis		Isolation of products
				Titration	Van Slyke method	
	mg. N per cc.		hrs.	per cent	per cent	
Carbobenzoxylglycyl- glycine (4)*	0.510	7.5	24		30	
			48		50	
Carbobenzoxylglycyl- <i>L</i> - alanine (5)	0.083	7.3	1	66		
			2	81		
			10	102		
Carbobenzoxylglycyl- <i>L</i> - phenylalanine	0.00037	7.3	1	58	58	Carbobenz- oxylglycine
			2	80	82	
			4	87		
			24	103	93	
Carbobenzoxylglycyl- <i>L</i> - tyrosine (6)	0.00037	7.7	1		37	<i>L</i> -Tyrosine
			2		†	
Carbobenzoxyl- <i>L</i> - glutamyl- <i>L</i> -phenyl- alanine (7)	0.00037	7.4	1	13		
			2		19	
			6	31		
			7		37	
			24	61	61	
Carbobenzoxyl- <i>L</i> - glutamyl- <i>L</i> -tyrosine (8)	0.00037	7.4	1		5	<i>L</i> -Tyrosine
			3		21	
			6		36	
			7		†	
Carbobenzoxyl- <i>L</i> - glutamyl- <i>L</i> -phenyl- alanineamide*	0.520	7.9	24		0	
			48		5	
$\alpha$ -Hippuryl- $\epsilon$ -carbobenz- oxyl- <i>L</i> -lysine (9)*	0.520	7.4	24		8	
			48		8	
$\alpha$ -Hippuryl- <i>L</i> -lysine	0.208	7.4	1	23		Hippuric acid
			3	52		
			7	79		
			24	91		
<i>L</i> -Tyrosyl- <i>L</i> -tyrosine (8)	0.072	7.5	24		†	
	0.520	7.5	1		†	<i>L</i> -Tyrosine†
Glycyl- <i>L</i> -tyrosine	0.645	7.5	4		11	
			24		†	"
Benzoylglycylglycine	0.520	7.2	5		64	Hippuric acid
			24		80	
Benzoyl- <i>L</i> -phenylal- anine*	0.520	7.2	24		20	
			72		34	
Carbobenzoxylglycyl- <i>L</i> - glutamic acid*	0.510	7.4	24		12	
			72		38	
Chloroacetyl- <i>L</i> -tyrosine	0.00037	7.5	24		1	

\* Temperature 25°.

† Tyrosine crystallization.

‡ NH<sub>2</sub>-N, found 7.6 per cent.

significance is the fact that the other hydrolytic product, the chloroacetic acid, has an inhibiting effect upon the enzyme (see Table II). Consequently, the splitting of chloroacetyl-*l*-tyrosine by carboxypeptidase does not follow the course of a first order reaction. On the other hand, the hydrolysis of carbobenzoxyglycyl-*l*-phenylalanine was found to accord rather closely with

TABLE II  
*Inhibition of Crystallized Carboxypeptidase by Chloroacetic Acid and Formaldehyde*

Substrate	Carboxypeptidase, 3 times crystallized	pH	Temperature	Time	Hydrolysis		
					No addition	Chloroacetic acid*	Formaldehyde†
	mg. N per cc.		°C.	hrs.	per cent	per cent	per cent
Chloroacetyl- <i>l</i> -phenylalanine (11)	0.0039	7.2	40	3			2
				24			5
	0.0039	7.5	25	1	25	10	13
				2	36	18	15
				3	47	24	17
Carbobenzoxycycyl- <i>l</i> -alanine	0.0720	7.3	40	3	73		2
				24	100		0
Carbobenzoxycycyl- <i>l</i> -phenylalanine	0.00037	7.4	40	3	83		0
				24	94		4
	0.00024‡	7.6	25	1	16	9	4
				2	32	17	3
				3	45	26	3

\* 0.05 mm sodium chloroacetate per cc. was added to the substrate solution before the enzyme.

† 0.2 cc. of 40 per cent formaldehyde (Merck) per cc. was added to the substrate solution before the enzyme.

‡ This enzyme solution was kept in the ice box for several weeks previous to its use and thus had lost some of its activity.

the kinetics of a first order reaction, as illustrated in Table III. The rate of reaction is approximately proportional to the enzyme concentration.

In view of the foregoing, carbobenzoxyglycyl-*l*-phenylalanine represents a very suitable substrate for determinations of carboxypeptidase activity. 1 carboxypeptidase unit may be defined as that quantity of enzyme which, when dissolved in 1 ml. of a solution containing 0.05 mm of carbobenzoxyglycyl-*l*-phenylalanine,

under standard conditions<sup>1</sup> causes a hydrolysis with a reaction constant of  $K = 0.0020$ , where  $a$  is the initial concentration of substrate,  $a - x$  is the concentration of substrate at time  $t$  (in minutes), and  $K = 1/t \log a/(a - x)$ .

The values reported in Table III demonstrate that our four times crystallized enzyme preparation had an activity corresponding to 6173 carboxypeptidase units per mg. of N. 1 unit was contained in about 1.1  $\gamma$  of our crystalline carboxypeptidase.

TABLE III

*Hydrolysis of Carbobenzoylglycyl-L-Phenylalanine by Crystalline Carboxypeptidase at 25°*

Carboxypeptidase, 4 times crystallized; pH 7.5	Time	Hydrolysis*	$K = \frac{1}{t} \log \frac{a}{a-x}$
mg. N per cc.	min.	per cent	
0.000097	60	15	0.0012
	120	28	0.0012
	180	40	0.0012
0.000194	30	15	0.0024
	60	29	0.0025
	90	41	0.0025
	120	49	0.0024
	180	63	0.0024
	240	77	0.0027
0.000388	30	32	0.0056
	60	53	0.0055
	90	68	0.0055
	120	77	0.0053

\* Estimated in 1 cc. samples by  $\text{NH}_2\text{-N}$  determination.

In studies of the stability of carboxypeptidase under various conditions, the enzymatic activity is usually tested at the beginning and at the end of each treatment. In tests of this kind it is essential to begin each experiment with an enzyme concentration so low that a decrease of the enzyme concentration during the experiment must result in a decrease of the speed of the enzymatic hydrolysis. The initial enzyme concentration must be a different one for each synthetic substrate, because of the

<sup>1</sup> See "Experimental."

fact that the various substrates differ widely in their sensitivity toward carboxypeptidase. Failure to take this important factor into account may lead to erroneous conclusions. Abderhalden and Abderhalden (2) have found that a solution containing 1 per

TABLE IV

*Partial Inactivation of Crystalline Carboxypeptidase*

Two solutions, A and B, of twice crystallized carboxypeptidase were kept at 40° and tested for activity by means of the hydrolysis of synthetic substrates at regular intervals in the usual manner.

Experiment No.	Substrate	Initial enzyme concentration	Time of inactivation at 40°	pH	Hydrolysis	
					During 1 hr. at 40°	During 2 hrs. at 25°
Solution A, 1.04 mg. N per cc. in M/3 disodium phosphate; pH 8.3						
1	Carbobenzoxylglycyl- <i>l</i> -alanine	0.083	0	7.3	67	
			1		46	
			2		30	
2	Chloroacetyl- <i>l</i> -tryptophane	0.016	0	7.3	65	
			1		38	
			2		24	
3	"	0.083	0	7.4	94	
			2		88	
Solution B,* 0.0243 mg. N per cc. in 5 per cent sodium chloride; pH 7.5						
4	Chloroacetyl- <i>l</i> -phenylalanine	0.0049	0	7.6		45
			1			29
			2			19
5	Carbobenzoxylglycyl- <i>l</i> -phenylalanine	0.00024	0	7.6		40
			1			24
			2			14
6	"	0.0049	2	7.5		98

\* This enzyme solution was kept in the ice box for several weeks before its use and thus had lost some activity.

cent of "carboxypeptidase," when kept at 56°, became inactive toward  $\beta$ -naphthalenesulfonylglycyl-L-tyrosine much sooner than toward chloroacetyl-DL-leucine. This finding was interpreted as indicating that the two substrates are split by two different enzymes.

It will be apparent from Experiments 1 and 2 of Table IV that, when the initial enzyme concentrations are adapted to the sensitivity of the substrates, no indication of an enzymatic inhomogeneity can be observed. It will be noticed that at 40° the activities toward chloroacetyl-*L*-tryptophane and carbobenzoxyglycyl-*L*-alanine are destroyed at about identical rates.<sup>2</sup> A comparison of Experiments 1 and 3, however, shows that, when identical initial enzyme concentrations are employed for both substrates, the activity toward carbobenzoxyglycyl-*L*-alanine disappears much more rapidly. In Experiment 3 the initial enzyme concentration represents a huge excess.

In Experiments 4 to 6 the inactivation was effected under slightly different conditions. Chloroacetyl-*L*-phenylalanine and carbobenzoxyglycyl-*L*-phenylalanine were employed as substrates. When the appropriate initial enzyme concentrations were employed, the rate of enzyme destruction was approximately the same for the two substrates; if identical enzyme concentrations were used for both substrates, as in Experiments 4 and 6, the activity toward chloroacetyl-*L*-phenylalanine appeared to be destroyed more rapidly.

In the last analysis, all comparative enzyme experiments performed with the aid of simple substrates consist in a comparison of reaction velocities. Therefore, the initial enzyme concentration must be sufficiently low so that the observed hydrolysis can be taken as a true expression of the quantity of active enzyme. Experiments 3 and 6 of Table IV and the previously discussed experiments of Abderhalden do not satisfy this condition, and therefore cannot be interpreted as indicating an enzymatic inhomogeneity of crystalline carboxypeptidase.

With the use of various synthetic substrates, the action of formaldehyde upon carboxypeptidase has been studied. The experiments reported in Table II demonstrate that formaldehyde inactivates carboxypeptidase at 40° and also at 25°.

The authors wish to express their thanks to Mr. Stephen M. Nagy who performed the analyses reported in this paper.

<sup>2</sup> At 40° the destruction of carboxypeptidase proceeds more slowly than at 56°.

## EXPERIMENTAL

*Carbobenzoxyglycyl-l-Phenylalanine*—To an ice-cold ethereal solution of *l*-phenylalanine ethyl ester (prepared from 3.8 gm. of the hydrochloride) there were added, in two portions, 3.8 gm. of carbobenzoxyglycyl chloride dissolved in ether, the second portion being followed by 50 cc. of a saturated aqueous solution of potassium bicarbonate. The mixture was shaken for 30 minutes and kept at room temperature for 1 hour. 2 cc. of pyridine were then added, and the reaction mixture was worked up in the usual manner. On saponification, the corresponding oily ester yielded 4 gm. of crystals which were purified by crystallization from a mixture of ethyl acetate and ether. M.p., 125–126°.

$C_{19}H_{20}O_6N_2$	Calculated.	C 64.0, H 5.6, N 7.8
356.4	Found.	" 63.7, " 5.6, " 7.7
$[\alpha]_D^{25} = +38.5^\circ$ (5% in ethyl alcohol)		

178 mg. of this compound were hydrolyzed by carboxypeptidase. The hydrolysate was acidified to Congo red, filtered, and concentrated to a small volume *in vacuo*. The concentrate was then extracted several times with ethyl acetate. The combined ethyl acetate extracts were washed with water, dried over  $Na_2SO_4$ , and concentrated, yielding 100 mg. of crystals (96 per cent of the theory) which were purified by crystallization from a mixture of ether and petroleum ether. M.p., 120–121°. The mixed melting point with carbobenzoxyglycine was 120–121°.

$C_{10}H_{11}O_4N$  (209.2). Calculated, N 6.7; found, N 6.7

*Estimation of Carboxypeptidase*—To 445 mg. of carbobenzoxyglycyl-*l*-phenylalanine are added 2.5 cc. of M/3 phosphate buffer of pH 7.6, 1.25 cc. of N sodium hydroxide, and enough water to make the total volume 10 cc. 2 cc. of the above substrate solution are mixed with the carboxypeptidase solution to be tested, and the volume is made up to 5 cc. with water. The reaction mixture is kept at 25° and 1 cc. aliquots are withdrawn for amino nitrogen estimation at the beginning and at 1 and 2 hour intervals. 100 per cent hydrolysis of the substrate corresponds to an increase of 0.7 mg. of  $NH_2-N$ . In the performance of such an estimation, the enzyme concentration in the test solution should

be so adjusted that, under the above conditions, the extent of hydrolysis in 1 hour is 10 to 30 per cent.

*Carbobenzoxyglycyl-l-Glutamic Acid*—An ethyl acetate solution of *l*-glutamic acid diethyl ester (prepared from 2.4 gm. of the hydrochloride) was coupled with 1.1 gm. of carbobenzoxyglycyl chloride, as described for carbobenzoxyglycyl-*l*-phenylalanine. The corresponding ester (2.9 gm.) was dissolved in 20 cc. of *N* NaOH by the addition of a few cc. of methanol, and allowed to stand for 2 hours. The solution was then acidified to Congo red with *N* HCl and concentrated to a small volume *in vacuo*. The crystals which had separated out were filtered off and purified by crystallization from hot water. M.p., 160–162°; yield, 1.2 gm.

$C_{15}H_{15}O_7N_2$ .	Calculated.	C 53.3, H 5.4, N 8.3
338.3	Found.	" 53.3, " 5.4, " 8.1
$[\alpha]_D^{25} = +4.0^\circ$ (5% in ethyl alcohol)		

*Carbobenzoxy-l-Glutamyl-l-Phenylalanineamide*—1 gm. of carbobenzoxy-*l*-glutamyl-*l*-phenylalanine ethyl ester (7) was dissolved in 100 cc. of methanol which had previously been saturated with dry ammonia. The solution was kept 3 days at room temperature and was then concentrated *in vacuo*. The residue was taken up in water and acidified to Congo red with *N* HCl. The crystals which separated out were filtered off, dried, and recrystallized from methanol. Needles which melted at about 185–187° were obtained. Yield, 500 mg.

$C_{22}H_{23}O_4N_2$ .	Calculated.	C 61.8, H 5.9, N 9.8
427.4	Found.	" 61.6, " 5.8, " 9.9

*$\alpha$ -Hippuryl-l-Lysine*—2 gm. of  *$\alpha$* -hippuryl- $\epsilon$ -carbobenzoxy-*l*-lysine (9) were dissolved in 20 cc. of methanol and hydrogenated in the presence of a palladium catalyst. The catalyst was then filtered off and the filtrate concentrated *in vacuo*, yielding a syrup which soon crystallized. The material, which was purified by crystallization from a mixture of water and dioxane, consisted of needles that melted at 236–238°. Yield, 1 gm.

$C_{11}H_{11}O_4N_2$ .	Calculated.	C 58.6, H 6.9, N 13.6
307.4	Found.	" 58.6, " 6.8, " 13.5 (Dumas)
$[\alpha]_D^{25} = -5.2^\circ$ (2.5% in $H_2O$ )		



192 mg. of this compound were hydrolyzed by carboxypeptidase. The hydrolysate was acidified, with Congo red as indicator, and evaporated *in vacuo*. The crystals thus obtained were recrystallized from hot water and melted at 188–190°. Yield, 93 mg. (83 per cent of the theory). The mixed melting point with hippuric acid was 188–190°.

$C_9H_9O_3N$  (179.2). Calculated, N 7.8; found, N 7.7

*Benzoylglycylglycine*—This compound was prepared as described by Fischer (12).

118 mg. of benzoylglycylglycine were hydrolyzed by carboxypeptidase. 76 mg. (85 per cent of the theory) of hippuric acid were isolated from the hydrolysate in the manner previously described under the splitting of  $\alpha$ -hippuryl-*L*-lysine. The product melted at 188–190°. The mixed melting point with hippuric acid was 188–190°.

$C_9H_9O_3N$  (179.2). Calculated, N 7.8; found, N 7.7

### *Enzymatic Studies*

The crystalline carboxypeptidase was prepared and recrystallized according to the directions of Anson (3). The substrates containing a carboxyl group were used in the form of their sodium salts. The concentration of the substrates was 0.05 mm per cc. in all cases. The solutions were buffered by m/30 phosphate buffers. All the pH values were measured by the glass electrode. Except where otherwise stated, the temperature was 40°. The extent of hydrolysis was followed either by determining the amino nitrogen liberated in the Van Slyke microvolumetric apparatus or by measuring the liberated carboxyl groups according to the method of Grassmann and Heyde (13). Enzyme blanks and tests for the lability of the substrate in the absence of carboxypeptidase were performed.

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## ACTIVATION OF ENZYMES

### VI. PURIFIED LIVER ARGINASE: REVERSIBLE INACTIVATION AND REACTIVATION

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Although previous studies in this laboratory (1, 2) and elsewhere (3-5) have suggested in the action of arginase the participation of a metal ion, the evidence in favor of this hypothesis heretofore has been of an indirect character, based in part upon observations of an increased rate of argininolysis in the presence of certain divalent ions. A seemingly less indirect support for the assumption was the finding of Edlbacher and Baur (4) that the activity could be greatly decreased by prolonged dialysis of their preparations and then restored by the addition of certain ions, of which the most effective was apparently  $Mn^{++}$ . However, the concentration of  $Mn^{++}$  required for the restoration of even a small part of the activity was about 100 times greater than that found by us with purified arginase (see below). The above authors found  $Mn^{++}$  in both dialysate and protein fractions and apparently tested for the presence of no other metals. Their conclusion that  $Mn^{++}$  is the metal ion involved seems therefore somewhat premature, especially in view of the observation recorded below that  $Co^{++}$  in a concentration as low as the smallest found effective with  $Mn^{++}$  can under suitable conditions restore to reversibly inactivated enzyme even more activity than does the latter ion.

Since the crude liver extracts generally used as a source of arginase contain a large number of metals, many of which presumably are coordinated to a greater or lesser extent with the

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various proteins, it seemed to us that little conclusive information as to the identity of the metallic constituent of arginase could be obtained until there be effected a separation of the enzyme from the excess of inactive substances present in these extracts. Therefore our first object in this investigation was the isolation of the enzyme. There has been achieved a partial purification; our best preparation was over 100 times as active as the liver powder used by Hellerman and Perkins (1). Direct spectroscopic analysis of the ash of this preparation as well as studies of its reversible inactivation and reactivation have given further evidence that arginase contains  $Mn^{++}$  (or possibly<sup>1</sup>  $Fe^{++}$ ) as an essential metallic constituent.

#### EXPERIMENTAL

The composition and designation of the buffers used in testing the enzyme and in its isolation and inactivation are given in Table I.

*Method of Determination*—The method used was essentially that described by Hellerman *et al.* (1, 2), with the following alterations. The reaction mixture consisted of 5 ml. of buffer, No. 7.5 P, 1 ml. of enzyme solution, and 1 ml. of 0.09 M arginine hydrochloride or other substrate, with further additions as indicated, in a total volume of 9 ml. After the reaction had been stopped in a boiling water bath, 8 mg. of NaCN and 0.1 gm. of crude urease were added and the solution left for only 15 minutes at 37° before aeration. Under these conditions, the blank due to the arginase activity in crude urease is less than 0.1 ml. of 0.02 N HCl. Since the enzyme was more active and the metal effects more reproducible with a more dilute buffer, the concentrated Buffer 7.5 P was replaced by 5 ml. of Buffer 7.6 P in some of the later experiments. However, the NaCN had then to be neutralized to avoid a large rise in pH with consequent increase in the blank; 0.5 ml. of a 1:1 mixture of 1 M  $KH_2PO_4$  and 1.6 per cent NaCN was therefore added in place of NaCN alone. Reaction mixtures containing more alkaline buffers (used in a few experiments) were neutralized with HCl before the addition of buffered cyanide.

<sup>1</sup> See under "Discussion."

Hellerman and Perkins (1) found that the enzymatic hydrolysis of arginine followed under well defined conditions the course of a first order reaction. On this basis, the extent of hydrolysis in 2 hours at 37° was measured with varying amounts of enzyme. It was found that when the rate of the reaction was expressed as  $k = \frac{1}{2} \log a/(a - x)$ , where  $a = \text{NH}_3$  produced when the reaction is allowed to go to completion and  $x = \text{NH}_3$  found in 2 hours,  $k'$  was directly proportional to the concentration of enzyme for amounts causing between 10 and 75 per cent hydrolysis in Buffer 7.5 P (Table I). When there were present 1.5 mg. of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$

TABLE I  
*Composition of Buffers*

Buffer No.	Composition	pH
4.3 Ac	$\text{HC}_2\text{H}_3\text{O}_2$ , 1.2 M; $\text{NaC}_2\text{H}_3\text{O}_2$ , 0.5 M	4.3 (on dilution 1:5)
5.4 Ac	" 0.084 M; " 0.5 "	5.4 ( " " 1:5)
7.0 P	$\text{KH}_2\text{PO}_4$ , 0.5 M; $\text{NaOH}$ , 0.295 M	7.0 ( " " 1:10)
N. P.	" 0.002 M; $\text{K}_2\text{HPO}_4$ , 0.002 M	
7.5 P	" 0.633 " " 0.1026 M	7.46 (in test mixture)
7.6 P	" 0.0569 " " 0.00923 M	7.53 " " "
9.2 G	Glycine, 0.126 " $\text{NaCl}$ , 0.126 M; NaOH, 0.054 M	9.20 " " "

The pH of the first three buffers was calculated from their composition (*cf.* (6) pp. 200-220) and checked colorimetrically. The pH of the last three was measured with the shaking hydrogen electrode under the conditions used in the test (5 cc. of buffer, 1 cc. of 0.09 M arginine hydrochloride, and 3 cc. of water) except that the temperature was 30°.

or 1.3 mg. of  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $k'$  was of course greater, but still increased linearly with the enzyme concentration. Therefore,  $k'$ , measured with 1 ml. of diluted enzyme in the test, divided by the dilution, is proportional to the concentration of enzyme ( $= [E]$ ) in the original solution and  $[E]$  times the volume is a measure of the total quantity denoted as  $U$ .  $U_{\text{Co}}$  is calculated in the same way from  $k'_{\text{Co}}$ , the rate with 1.5 mg. of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  in the test mixture, and represents the total activity in the presence of excess  $\text{Co}^{++}$ . At each stage in the purification of liver arginase the nitrogen content was determined by the micro-Kjeldahl procedure. We use as an indication of purity  $U:N$ , where

$N$  = total mg. of nitrogen. The amount of enzyme,  $U$ , is always given in terms of its activity in Buffer 7.5 P.

### *Purification of Arginase*

Unless otherwise stated, all dialyses were carried out against Buffer N. P. at  $0^{\circ}$ . All precipitates were separated by centrifugation in a cold room at approximately  $10^{\circ}$  and then dissolved in water and enough Buffer 7.0 P to make the final phosphate concentration 0.01 to 0.02 M. The yields are given for each separate step.

Fresh ground beef liver was stirred with 1 volume of water, the mixture pressed through muslin, filtered, and the filtrate treated with acetone (1, 2). The precipitated, acetone-washed, dried powder was extracted four times with sufficient water to make a final total volume in ml. 20 times the number of gm. of original weight. This solution contained on the average 76 per cent of the activity present in the liver extract;  $U:N$ , 0.7 to 0.8.

*Acetone Fractionation*—It was found advisable to test on small portions of the above solution the exact amounts of acetone necessary for the best yield of enzyme. Generally 0.50 volume of acetone was added, the inactive precipitate discarded, and a further 0.36 volume of acetone added to the supernatant to precipitate the enzyme. The suspension was allowed to stand 2 to 3 hours between the addition of acetone and centrifugation in each step. The whole procedure was always carried out in 1 day at a temperature not exceeding  $10^{\circ}$ .

The active precipitate was dissolved in about an equal volume of water as soon as possible and dialyzed overnight. It was then heated in a water bath to  $56$ – $58^{\circ}$  and kept at this temperature for 3 minutes, cooled in ice, and centrifuged to remove denatured protein. Yield, 67 per cent;  $U:N$ , 1.9.

From one later experiment it appears that the acetone *fractionation* may be performed directly on the initial crude liver extract, with avoidance of the tedious drying with acetone. In this case 0.4 volume of acetone was added to precipitate inactive proteins, and 0.23 volume of acetone per volume of supernatant was used to precipitate the enzyme. Yield, 67 per cent;  $U:N$ , 1.9.

*Ammonium Sulfate Fractionation*—The solution from the above step was brought to 0.42 saturation with  $(\text{NH}_4)_2\text{SO}_4$  by addition

of 0.73 volume of a saturated solution and left 4 to 24 hours at 0–10°, after which it was centrifuged. The precipitate, containing 5 to 20 per cent of the enzyme, was discarded, and the arginase precipitated by addition of 11 gm. of  $(\text{NH}_4)_2\text{SO}_4$  to each 100 ml. of supernatant. The second precipitate was dialyzed for about 15 hours. Yield, 50 to 80 per cent, average 67 per cent;  $U:N$ , 2 to 5.

*Salicylate Treatment*—Although this step often leads to no apparent improvement in purity, owing partly to a considerable destruction of enzyme, it is helpful in the removal of deeply colored impurities and in facilitation of further purification upon repetition of the ammonium sulfate step. The following reagents were added in the order given to the ice-cold solution ( $[E] = 20$ ), 0.091 volume of Buffer 7.0 P, 0.64 volume of 2 M sodium salicylate, and, after 2 to 3 minutes, 0.64 volume of saturated  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate of denatured protein was removed at once by centrifugation for 20 minutes in the cold and the supernatant fluid dialyzed for about 20 hours. 26 gm. of  $(\text{NH}_4)_2\text{SO}_4$  were then added for each 100 ml., the precipitate discarded, and 11 gm. of  $(\text{NH}_4)_2\text{SO}_4$  added to the supernatant. The second precipitate was dialyzed for 15 to 20 hours. Yield, 33 to 53 per cent;  $U:N$ , 2.9 to 7.2 (generally 3 to 3.5).

The enzyme again was heated to 57° for 4 minutes and fractionated with  $(\text{NH}_4)_2\text{SO}_4$  between 0.43 and 0.57 saturation by means of the addition of suitable amounts of a saturated solution. Yield, 53 to 87 per cent;  $U:N$ , 3.4 to 6.5.

The heating and ammonium sulfate fractionation steps were repeated until the solution was pale yellow in color and  $U:N$  was 5 or greater. When  $U:N$  is larger than 4, it is advisable to have present 0.02 M phosphate buffer of pH 7 to avoid partial inactivation through the acidity of the ammonium sulfate. The buffer must not be added before heating, since in the presence of added phosphate (or other salt) arginase is partially destroyed at 57°, although it is quite stable at 57° in the absence of added salt.

*Fractionation with Sodium Alizarin Sulfonate*—Buffer 5.4 Ac, 0.2 volume, and 0.1 to 0.3 volume of 4 per cent sodium alizarin sulfonate (the exact amount necessary must be determined by preliminary tests) were added to the enzyme solution kept in an ice bath. The suspension was centrifuged after  $\frac{1}{2}$  to 1 hour; the



precipitate was dissolved with Buffer 7.0 P and water, and the solution centrifuged to remove any insoluble material, after which the enzyme was reprecipitated by the addition of 0.2 volume of Buffer 5.4 Ac and sufficient 0.2 N acetic acid to bring the pH of the phosphate present to 5.4. The precipitate was dissolved to a volume such that  $[E]$  equaled 11, and then 0.05 volume of Buffer 7.0 P was added. This solution was fractionated with ammonium sulfate between 0.33 and 0.57 saturation. The first  $(\text{NH}_4)_2\text{SO}_4$  precipitate contained most of the alizarin sulfonate and sometimes as much as 30 per cent of the enzyme; the latter could be recovered, after removal of the alizarin sulfonate by dialysis, by means of refractionation with  $(\text{NH}_4)_2\text{SO}_4$ . The main part of the arginase, in the precipitate at 0.5 saturation, was dialyzed first against Buffer N. P. for 1 day, and then for 2 days against conductivity water. The enzyme at this final stage was still colored by non-dialyzable alizarin sulfonate, for which there was found no convenient means of removal. This did not seem to interfere with the enzyme activity. Yield, 47 to 67 per cent;  $U:N$ , 13.5 to 18.

The over-all yield was about 5 per cent and  $U:N$  50 to 65 times greater than in the crude extract ( $U:N$ , 0.27).

### *Reversible Inactivation*

The procedures used (3, 4) by other workers for reversible inactivation did not seem altogether suitable for the purpose of separating protein and metal ion. A treatment with HCl at pH 3 (3) is too rapidly destructive of enzyme and greatly prolonged dialysis (4) is inconvenient. The most successful method found by us was the following. Of Buffer 4.3 Ac, 0.2 to 0.4 volume was added to the ice-cold enzyme solution and the mixture left 12 hours at  $0^\circ$ ; then enough saturated  $(\text{NH}_4)_2\text{SO}_4$  was added to bring the solution to 0.57 saturation. 1 hour later the suspension was centrifuged at  $10^\circ$ , and the precipitate washed once with 0.57 saturated  $(\text{NH}_4)_2\text{SO}_4$ , dissolved in dilute phosphate buffer, and either tested at once or dialyzed overnight, first against Buffer N. P. and then conductivity water. This procedure was not completely satisfactory in that it gave rather variable results with different preparations and in no case removed all activity (the latter being based on tests in the absence of added metal ion); but if buffers of greater acidity were used there resulted irreversible inactivation, all action, even with added  $\text{Co}^{++}$ , being

destroyed. The difficulty probably lay in the following: at acidities greater than pH 5.0, the protein is denatured with increasing rapidity and the rate of dissociation of the metal is not sufficiently greater to allow complete separation of the two processes. It was possible to obtain an estimate of all the metals in one of these preparations before and after inactivation by a spectrographic method (7) which permits a semiquantitative determination of nearly all metals in one sample. The analyses were performed by Dr. M. Slavin of the Bureau of Mines and the authors wish to express here their gratitude for his cooperation.

TABLE II

*Spectroscopic Analysis of Purified Arginase and Derived Inactivated Preparations*

Enzyme No.....	9A	9R	10A	10R2
U, before inactivation	283	283	267	267
U <sub>Co</sub> " "	1060	1060	1500	1500
U, after " "		30		104
U <sub>Co</sub> " "		1040		1500
Cu	Present	Present	4.6 $\gamma$	2.2 $\gamma$
Fe	"	"	40 $\gamma$	23 $\gamma$
Mg	"	"	16 $\gamma$	3.5 $\gamma$
Mn	"	Trace	7.7 $\gamma$	2.2 $\gamma$

Enzymes 9A and 10A were purified arginase preparations from which, respectively, Enzymes 9R and 10R2, partially inactivated preparations, were derived by the procedure described in the text. The amount of Mn in Enzyme 9A was estimated as roughly 10 times greater than that in 9R.

U is a measure of the total quantity of arginase; U<sub>Co</sub>, of the total activity in the presence of excess Co<sup>++</sup>. See "Method of determination."

The complete analysis by separate chemical methods would have been impossible owing to the prohibitive quantity of material which would have been required. The samples were ashed in platinum crucibles, additions being 23 mg. of NaCl as a diluent and 0.5 cc. of H<sub>2</sub>SO<sub>4</sub> to aid in the destruction of organic matter. A sample of Buffer N. P. was treated similarly and taken as a blank. Unfortunately, in the case of Enzyme 9, which on treatment with acetate and ammonium sulfate yielded a preparation, Enzyme 9R, which retained only a ninth of the initial activity (alone), but was completely reactivatable in the presence of Co<sup>++</sup> (cf. Table II, second and third columns), the ash was so hygroscopic, owing to

TABLE III  
Effect of Various Metals on Acid-Treated Arginase

Enzyme No.	Metal added	Amount of metal in test	Other additions	k' of original enzyme	k' after inactivation	k' after reactivation	Amount of metal in original
		$\gamma$					$\gamma$
10R7	Mn <sup>++</sup>	0.005		0.160	0.093	0.090	0.005
	"	0.011		Same	Same	0.132	Same
	"	0.022		"	"	0.155	"
	"	0.043		"	"	0.266	"
	"	0.54		"	"	0.284	"
10R6	"	0.011		0.189	0.038	0.076	0.005
	"	0.043		Same	Same	0.124	Same
	Cu <sup>++</sup>	0.006		"	"	0.033	0.003
	"	0.024		"	"	0.033	Same
	Fe <sup>++</sup>	0.06		"	"	0.025	0.03
10S	"	0.024		"	"	0.025	Same
	Mg <sup>++</sup>	0.06		"	"	0.025	0.01
	Mn <sup>++</sup>	0.011		0.188	0.029	0.060	0.005
	{ Mn <sup>++</sup>	{ 0.011	Fe powder	Same	Same	0.057	{ 0.005
	{ Fe <sup>++</sup>	{ 0.061					{ 0.028
11R2	Fe <sup>++</sup>	0.06	Cysteine	"	"	0.029	0.028
	"	0.24	"	"	"	0.058	Same
	"	0.24	Fe powder	"	"	0.049	"
	Mn <sup>++</sup>	0.01		0.269	0.092	0.201	0.006
	"	0.04		Same	Same	0.560	Same
11R3	Co <sup>++</sup>	0.01		"	"	0.528	0.000
	Mn <sup>++</sup>	0.01		0.278	0.114	0.223	0.006
	Fe <sup>++</sup>	0.036	Fe powder	Same	Same	0.148	0.03
11R4	"	0.121	" "	"	"	0.155	Same
	"	0.08		0.278	0.098	0.136	0.03
	{ Fe <sup>++</sup>	{ 0.04	Fe powder	Same	Same	0.164	{ 0.03
	{ Mn <sup>++</sup>	{ 0.006					{ 0.006

Tests on Enzymes 10R7, 10R6, and 10S were carried out with phosphate buffer No. 7.5 P ( $\mu = 1.1$ ); those on Enzymes 11R2, 11R3, and 11R4, with phosphate buffer No. 7.6 P ( $\mu = 0.1$ ). The metal estimated present in the untreated enzyme (last column) on the basis of enzymic activity and the analysis of Enzyme 10A is corrected in the tests involving Buffer 7.6 P to take account of the increased rate (one-third greater) in the dilute as compared with the concentrated buffer. Cysteine, where used, was added as the neutralized hydrochloride, 0.01 mg. being present in the test. Fe powder was a "c.p. reduced iron."

the use in this instance of KCl as diluent, that it could not be weighed. Qualitatively the intensity of the various spectral lines indicated that of the various metals originally present only

Mn was appreciably lower in this inactivated enzyme preparation. A complete analysis was obtainable for Enzyme 10A and the acid-inactivated preparation Enzyme 10R2 derived from it. The results are given in Table II. According to Dr. Slavin, the absolute values may be in error by  $\pm 100$  per cent, but the relative accuracy for any one metal in different samples is much greater ( $\pm 10$  per cent). The results have been corrected for the blank, which was zero in the case of Mn, 5 per cent of the total amount in Enzyme 10A for Fe, 20 per cent for Cu, and 27 per cent for Mg. V, Mo, Al, Si, and Pt were also present, and the concentration of these decreased on acid treatment.

In studying the reactivation of acid-inactivated arginase by traces of metal ions, we noted that the maximum effect was obtainable only when enzyme and metal ion were mixed in a concentration at least 10 to 50 times greater than in the solution added in the test. The constituents could be mixed in the absence of buffer, in the presence of 0.02 M phosphate between pH 7 and 7.5, or in 0.02 M glycine between pH 8 and 9 without alteration of the extent of activation. Citrate, concentrated or alkaline phosphate, or any buffer of pH lower than 6 apparently prevented the addition reaction of metal ion and enzyme. The results of the reactivation experiments are shown in Table III. The values of  $k'$  are all given for dilutions corresponding to those used with unaltered enzyme, after any volume changes involved in the procedures used have been taken into account. The last column contains an estimate, based upon the spectrographic analysis and activity of Enzyme 10A (Table II), of the amount of metal actually present before acid treatment in the quantity of enzyme used in the test.

#### *Hydrolysis of Canavanine and Several Other Guanidino Compounds*

The degree of hydrolysis of canavanine in the presence of similarly treated arginase preparations is given in Table IV and compared with that of L(+)-arginine under the same conditions. The activity is expressed as per cent hydrolysis in 2 hours at 37°, since the kinetics of the reaction are not known. The canavanine was prepared from jack bean meal (8); its purity was established by analysis<sup>2</sup> (calculated, C 34.07, H 6.86; found, C 34.1, H 6.6).

A few measurements were made with other selected guanidino

<sup>2</sup> We are grateful to Mr. William Saschek of Columbia University, who performed the microanalyses.

TABLE IV

*Comparative Activity of Purified Arginase toward l(+)-Arginine and Canavanine*

Enzyme No.	Metal in test		Per cent hydrolysis		pH	
			Arginine	Canavanine	Arginine	Canavanine
11S		$\gamma$	65	50	7.53	7.82
"	Mn <sup>++</sup>	1300	85	60	Same	Same
"	Co <sup>++</sup>	1500	96	80	"	"
11R3			33	30	"	"
11R2	Mn <sup>++</sup>	0.01	49	29	"	"
11R3	Fe <sup>++</sup>	0.08	47	27	"	"
11R3	{ Fe <sup>++</sup> Mn <sup>++</sup> }	{ 0.04 0.006 }	55	29	"	"
11K			54*	50	6.74	6.74
"			64	50	7.53	7.82
"			86	4.5	9.20	9.20

\* This value is calculated from the pH-activity curve of arginase-arginine, since the solutions of arginine and canavanine in this buffer differed in pH. They differ similarly for the buffer of pH 7.53 but no correction was necessary here, inasmuch as the activity toward arginine in phosphate buffers of  $\mu = 0.1$  changes negligibly between pH 7.4 and 7.9. The pH of the two substrate-buffer solutions was the same in the glycine buffer No. 9.2 G.

TABLE V

*Hydrolysis of Guanidino Compounds in Presence of Concentrated Purified Arginase*

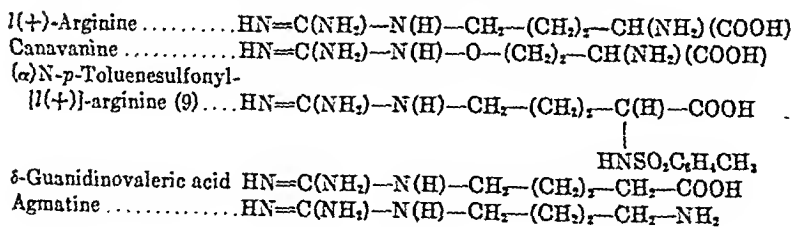
The figures represent per cent hydrolysis under the stated conditions at 37°.

Substrate	Buffer 7.6 P, 2 hrs.		Buffer 9.2 G, 2 hrs.		Buffer 9.2 G, 25 hrs.
	Alone	Plus Co <sup>++</sup>	Alone	Plus Mn <sup>++</sup>	Plus Mn <sup>++</sup>
Agmatine sulfate, 23.2 mg.	4	16	5	66	70
$\delta$ -Guanidinovaleric acid, 15.9 mg.....	0.6	3	0.5	21	94
p-Toluenesulfonylarginine, 20.6 mg.....	0.0	0.7	1	11	94*

\* Incubated 44 hours.

compounds in an attempt to determine further to what extent the apparent narrow specificity of arginase is a quantitative rather than a qualitative one. In each of these experiments there were present 170 times the concentration of purified arginase necessary to bring about in 2 hours at 37° 55 per cent hydrolysis of l(+)-arginine in Buffer 7.6 P, or 67 per cent in Buffer 9.2 G. The results in terms of per cent hydrolysis are given in Table V. Blanks in the absence of arginase, with all other conditions unchanged, were always less than 0.1 ml. of 0.02 N HCl for all substrates in phosphate buffer and for *p*-toluenesulfonylarginine and  $\delta$ -guanidinovaleric acid in the glycine buffer, 0.3 ml. for agmatine sulfate in the latter. Blanks in controls with added arginase without subsequent addition of urease in the runs with glycine buffer and added  $Mn^{++}$  were always less than those without arginase; this was taken to indicate that from these compounds, as from arginine, the enzyme forms urea and no ammonia (*cf.* (2)).

The identity of each substance used as a substrate was determined by its physical properties and by its analysis.<sup>2</sup> Of the materials used, only the agmatine was purchased on the market; after recrystallization, this remained slightly impure. The choice of substrates selected for study was determined by their structural relationship to arginine, as indicated in the following.



#### DISCUSSION

The rather large number of metals found in the spectrographic analysis of the ash of the best arginase preparation available showed that the purification had not yet proceeded far enough to permit a direct observation of any one metal naturally associated with the enzyme. The presence in blanks of most of these metals, in quantities by no means negligible, suggests the necessity ultimately of rigorous purification of all salts used in the isolation

procedure, since proteins tend to form difficultly separable complexes with many divalent metal ions. The latter applies especially in the less acid regions of pH in which it is necessary to operate with arginase, an enzyme exhibiting marked instability in slightly acid solution.

It was found that treatment at pH 4.3 removed from enzyme preparation No. 10A all of the metals to approximately the same extent. Nevertheless, from the spectroscopic analysis, taken together with data on the effect of added substances, it is possible to draw some conclusions. Of the several ions which have been reported (1, 3) to activate arginase,  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Zn}^{++}$ , and  $\text{Cd}^{++}$  were absent and vanadium was present in an amount no greater than in the blank. We found  $\text{Mg}^{++}$  and  $\text{Cu}^{++}$  in low concentrations to be wholly incompetent to activate; in higher concentrations  $\text{Cu}^{++}$  is known to inactivate markedly (1). There are no data on the effect of cuprous copper. Solid  $\text{Cu}_2\text{O}$  destroyed our enzyme completely, but this might have resulted from a partial oxidation to  $\text{Cu}^{++}$ . However, it seems unlikely that  $\text{Cu}^+$  could be involved in view of the observed insensitivity of purified arginase to such oxidizing agents as  $\text{K}_3\text{Fe}(\text{CN})_6$  and oxygen as well as to cyanide. The presence of 0.01 M  $\text{K}_3\text{Fe}(\text{CN})_6$  led only to a slight lowering of activity, differing but little from the effect of 0.01 M  $\text{K}_4\text{Fe}(\text{CN})_6$ . Furthermore, there was observed no effect from a treatment of the enzyme with 0.02 M cyanide at pH 8 for 12 hours, followed by precipitation of the enzyme with ammonium sulfate and its dialysis for 2 days against 0.01 M cyanide; such a procedure is known to remove completely the  $\text{Cu}^+$  from hemocyanins and polyphenol oxidase (10).

Of the other metals present, we found only  $\text{Mn}^{++}$ , and less consistently  $\text{Fe}^{++}$ , to be capable of restoring any activity to partially inactivated arginase (*cf.* Table III). The rate of hydrolysis as catalyzed by the acid-treated enzyme can be increased significantly by an amount of  $\text{Mn}^{++}$  equal in the test solution to 0.0012  $\gamma$  per cc. or  $2 \times 10^{-5}$  mm,<sup>3</sup> which is about twice that found in the purified arginase. By the addition of 4 times that amount of  $\text{Mn}^{++}$ , the rate can be raised *above* the level observed in the presence of the untreated purified enzyme.

<sup>3</sup> There is induced by  $\text{Co}^{++}$  added in this concentration an even greater activity (*cf.* Table III), in contrast to the finding of Edlbacher and Baur (4).

The results with  $\text{Fe}^{++}$  were somewhat ambiguous. In apparent contrast to the findings of some other workers (11-14) but in agreement with Edlbacher and Baur (4), large amounts (3 mg. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ), added directly to the test, were found to lead to no activation or reactivation with or without cysteine or other reducing agent. In some instances there resulted rather less activity. On the other hand, we have observed that the addition to the concentrated solution of amounts comparable to those present in unaltered enzyme, when there was added at the same time powdered iron or cysteine to prevent oxidation of the  $\text{Fe}^{++}$  to  $\text{Fe}^{+++}$ , did in many cases restore some activity. To obtain further evidence as to which of these two metals ( $\text{Mn}^{++}$  or  $\text{Fe}^{++}$ ) might normally be associated with arginase there was determined as a function of pH in phosphate and glycine buffers of ionic strength 0.1 the rate of hydrolysis of arginine in the presence of untreated, acid-inactivated, and  $\text{Fe}^{++}$ -,  $\text{Mn}^{++}$ -, and  $\text{Fe}^{++}$ - plus  $\text{Mn}^{++}$ -reactivated enzymes. The added metals were present in a concentration twice that found to be present in Enzyme 10A. None of the pH-activity curves, not even the one for acid-inactivated enzyme alone, was seen to coincide exactly with the original in shape. Neither of the metals was found to have any effect at pH 6.2 or below. However, the curves did show marked restoration of activity by  $\text{Mn}^{++}$  from pH 6.5 to 10.1, the maximum tested, and, in contrast, an increase in the rate, between pH 7.2 and 9.2 only, in the case of  $\text{Fe}^{++}$ , which, moreover, displayed much less effect than  $\text{Mn}^{++}$ .

In other respects also the reactivated enzymes differed from the original. Manganous ion, added in reactivations, was much more readily dissociated by dialysis or ammonium sulfate than the  $\text{Mn}^{++}$  in untreated arginase; and addition of neither  $\text{Fe}^{++}$  nor  $\text{Mn}^{++}$  in concentrations of  $1.4 \times 10^{-4}$  mM and  $2 \times 10^{-5}$  mM respectively led to a recovery of activity when canavanine was the substrate. These observations, and those showing that the addition to either normal, purified enzyme, or inactivated material, of  $\text{Mn}^{++}$  in amounts equivalent to from 2 to over 100 times the concentration of the metal originally present brings about continually increasing activity, might lead to a suspicion that metal activation is merely an artifact and that the acid treatment has caused only destruction of a large part of the enzyme. However, such a conclusion would seem unlikely in view of the



demonstration afforded in our procedure that the total activity in the presence of excess  $\text{Co}^{++}$  is either little changed or much less lowered than in the absence of any addition, and, furthermore, that a decrease in metal content is accompanied by a roughly proportional loss in activity. It appears more reasonable to hold to the hypothesis that a metal ion is an essential part of the arginase molecule; further, that the most probable essential metallic constituent is  $\text{Mn}^{++}$ , with  $\text{Fe}^{++}$  still a possibility either of itself or together with  $\text{Mn}^{++}$ .

Our purified enzyme itself appears to be capable of combining loosely and reversibly with excess  $\text{Mn}^{++}$  and with various other ions. In the hydrolysis of *l*(+)-arginine, and to a lesser degree of canavanine, this added metal is almost as effective "catalytically" (in the case of  $\text{Co}^{++}$  more so) as the metal originally present. At pH 4.3 the metal slowly dissociates from purified arginase and at the same time there are initiated in the protein changes leading eventually to its complete "denaturation;" the resulting enzymic material is now capable only of entering into the more readily reversible type of combination with  $\text{Mn}^{++}$ , supposedly either because the groups in the enzyme and other (inactive) proteins with which the metal initially might coordinate react more rapidly than the characteristic grouping in which it was originally tightly bound, or because the latter has been altered to some extent by the methods employed. That non-liver  $\text{Mn}^{++}$  was introduced during the isolation procedure is rendered unlikely by the absence of this metal from the buffer used for the dialyses (in contrast to all the other metals except non-activating Pb).

The experiments with substrates other than arginine and canavanine confirmed previous observations (2, 15-18) dealing with the relatively slight activity of arginase toward certain analogues of arginine in which the  $\alpha$ -amino or carboxyl group has been altered or removed. In phosphate buffer of pH 7.5, in the presence of concentrated arginase with or without cobalt, the hydrolysis of all three compounds listed in Table V (except only agmatine in the presence of  $\text{Co}^{++}$ ) was very slight; in glycine buffer of pH 9.2 the rates were hardly greater, but upon the addition of  $\text{Mn}^{++}$  they became appreciable, increasing in the order *p*-toluenesulfonyl-arginine,  $\delta$ -guanidinovaleric acid, and agmatine; in a prolonged incubation period there was observed nearly complete hydrolysis. The strikingly low rate with *p*-toluenesulfonylarginine is of interest

in view of the reports that the related compounds carbamido-*l*(+)-arginine (18), benzoylarginine (16), and acetylphenylalanyl-arginine (17) are hydrolyzed even in the absence of added metal ion, albeit by far more slowly than *l*(+)-arginine. The considerable increase observed upon addition of  $Mn^{++}$  in the rate of the enzyme-catalyzed hydrolysis at pH 9.2 of all of these compounds is of interest in connection with the theory (1, 2) that the rôle of the metal ion is related, at least in part, to its coordination with the  $\alpha$ -amino or carboxyl group (or both groups) of *l*(+)-arginine in the formation of intermediate enzyme-substrate complexes; in each of these substrates there has been eliminated one or the other of the characteristic groups. It seems altogether probable that the importance of the metallic constituent transcends the function of mere orientation (*e.g.*, through these groups). Moreover, the rate of hydrolysis of canavanine, in which occur both  $\alpha$ -amino and carboxyl groups but an altered (differently substituted) guanidino group, is increased to a lesser degree by either  $Mn^{++}$  or  $Co^{++}$ , especially when these are present in low concentrations, than is the rate for arginine. It may be remarked that in view of the very large decrease in the efficiency of catalysis by arginase observed when either of the substrate groups ( $\alpha-NH_2$ ;  $-COOH$ ) apparently unconcerned directly in the reaction has been altered (*cf.* the results on alkaline hydrolysis of guanidino compounds (2)) it is noteworthy that the substitution of an oxygen atom for the methylene group adjacent to guanidino, itself, results only in a slight alteration of the rate of hydrolysis, at least at pH 7.5, although this change in structure has occasioned a lowering of the association exponent,  $pK_a$ , of the guanidino group from between 13 and 14 (19) for arginine to 7.4 for canavanine (20, 21). It might be conceived that two different enzymes were involved. Due consideration is to be given the observations (Table IV) of the differing effects of small amounts of metal ions on the rates of hydrolysis for the two substrates as well as of the markedly different rates at pH 9.2. However, the latter observation is entirely consistent with the assumption that the more characteristic action of arginase is concerned only with a positively charged guanidinium ion (2). In arginine the guanidino group would be completely in the preferred ionic form throughout the pH range of enzyme activity with this substrate; but only 1.2 per cent of the cationic guanidinium form would be

present in the case of canavanine at pH 9.2 in the region where its enzymatic hydrolysis is remarkably slow.

## SUMMARY

1. A method for the partial purification of liver arginase is described.

2. Purified arginase, after inactivation by treatment with acetate buffer at pH 4.3 and precipitation with ammonium sulfate, has been shown to contain a lower concentration of all the metals originally present; of these only  $Mn^{++}$  and to a lesser extent  $Fe^{++}$  have been found capable of restoring activity. There are discussed certain differences between original and reactivated enzymes.

3. The relative rates of hydrolysis in the presence of purified arginase of *p*-toluenesulfonylarginine,  $\delta$ -guanidinovaleric acid, agmatine, and canavanine have been observed; differences in the behavior of the enzyme toward the last named compound and arginine have been noted.

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# GLYCOGEN, FAT, AND WATER CONTENT OF GUINEA PIG LIVER

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The object of this report is to present data upon the normal values of glycogen, fat, and water content of the livers of well nourished guinea pigs. Such data, essential as a basis for various types of experimental work, are not available in the literature for this species. As this study was getting under way, Petrén (1939) reported the results of an extensive investigation of the glycogen content of the guinea pig liver, from which he concluded that there were daily rhythmic variations, with a minimum in the morning (around 9 a.m.) and a maximum in the afternoon (around 3 p.m.). In order to check upon this finding, we have concentrated upon two periods of the day—9 a.m. and 3 p.m.—which are representative of the usual morning and afternoon experimental periods, and which, from Petrén's data, would be expected to show considerable differences in the mean value of the liver glycogen content. We have satisfied the following conditions in the present study: (1) all elements of the diet were available in excess at all times; (2) the animals were left entirely unmolested except for the daily cleaning of the cages and replenishment of the food supply.

## *Material and Methods*

Thirty-six male guinea pigs, subdivided into three series of twelve, were used for the experiments. We have used only male guinea pigs in order to avoid sex differences in liver glycogen, etc. (Deuel, Butts, Hallman, Murray, and Blunden, 1937, 1938; Sjögren, Nordenskjöld, Holmgren, and Möllerström, 1938). All

animals were obtained from the same breeding farm. Series 1, from 5½ to 6 months old, was received on May 22 and sacrificed on June 13 (Guinea Pigs 1 to 6) and 15 (Guinea Pigs 7 to 12). Series 2, 10 weeks old, was received on August 16 and sacrificed on September 26 (Guinea Pigs 13 to 18) and 28 (Guinea Pigs 19 to 24). Series 3, 10 weeks old, was received on October 17 and sacrificed on November 7 (Guinea Pigs 25 to 30) and 9 (Guinea Pigs 31 to 36). While in the laboratory, each series was housed in four small cages, three animals to a cage.

The diet consisted of Purina Rabbit Chow (complete ration), Purina Dog Chow (large checkers), fresh carrots, and lettuce. The Chows contain roughly 50 per cent carbohydrate, 20 per cent (Dog) and 14 per cent (Rabbit) protein, and 5 per cent (Dog) and 2.5 per cent (Rabbit) fat. When the two Chows are used together, the mineral and vitamin content is fully adequate except for vitamin C, which is supplied by the greens. The animals received no water, since they will not drink so long as fresh greens are available. The daily cleaning of the cages and replenishment of the food supply was deliberately carried out at different and irregular times on succeeding days.

At the time of sacrifice, the animals were removed from the four cages in rotation. After being weighed, they were quickly decapitated and held up by the hind legs for a brief interval, during which the mixed blood samples for sugar determination were collected. The liver was then rapidly exposed, and samples were taken in the following order: (1) for glycogen (from the lobe to the right of that to which the gallbladder is attached; where two samples were taken, the above sample is designated Sample A, and Sample B was taken from the lobe to which the gallbladder is attached); (2) for fat (from the extreme left lobe of the liver; where two samples were taken, the above sample is designated Sample A, and Sample B was taken from the extreme right lobe of the liver); (3) for water content (in Series 1, taken from the same lobe as the sample for fat; in Series 2 and 3, from the lobe to the right of, and ventral to, the lobe used for the fat sample); (4) for histological fixation for subsequent study (the total weight of these blocks was obtained by weighing the bottles of fixative before and after the addition of the tissue). All samples were dried quickly on filter paper before subsequent handling. The

remainder of the liver was then excised, the gallbladder removed, and this liver residue weighed. To obtain the total weight of the liver, the weight of all liver samples was added to that of the liver residue. Special experiments showed that the liver weight so obtained is only slightly less than that of the same liver weighed *in toto*, the difference being insignificant for the present study.

*Chemical Methods*—Blood sugar was determined on laked blood by the method of Folin and Malmros (1929). Glycogen was determined by the Good, Kramer, and Somogyi (1933) adaptation of Pflüger's method. We have added 0.5 cc. of saturated  $\text{Na}_2\text{SO}_4$  solution with the alcohol to insure precipitation of the glycogen, as recommended by Sjögren *et al.* (1938). For hydrolysis we have used 10 or 20 cc. aliquots of the glycogen solution in 1 N  $\text{H}_2\text{SO}_4$  and hydrolyzed for 2 hours on a water bath. That hydrolysis is complete under these conditions we have proved by duplicate determinations on smaller aliquots and by applying the technique to a similar solution of glycogen of known purity. For the determination of glucose in the neutralized hydrolysate we have used the method of Folin and Wu (Folin and Wu, 1920; Folin, 1929). The fat (total lipid) content of the liver was determined by the oxidation method of Bloor (1928), with the slight modifications of Marble, Field, Drinker, and Smith (1934). Total cholesterol was determined by the colorimetric method of Bloor (1928). Phospholipids were determined by the unpublished method of Fiske and Subbarow. All of the glycogen and most of the fat determinations were carried out in duplicate and sometimes in triplicate. The water content of the liver was determined by drying to constant weight at  $110^\circ$ . After removal from the animal, the tissue sample was transferred at once to a weighed, 50 cc. Erlenmeyer flask, tightly stoppered. Shortly thereafter the sample was rapidly minced in the flask with scissors, the weight of the minced sample determined, and the flask transferred to the oven.

### Results

The detailed data are given in Table I. The times are Eastern standard time (the divergent sacrifice time of the first three animals was occasioned by confusion with daylight saving time).

TABLE I  
*Analyses of Guinea Pig Livers*

Series No.	Guinea pig No.	Weight	Sacrificed at	Blood sugar	Liver weight		Liver glycogen	Liver fat	Liver H <sub>2</sub> O
		gm.		mg. per cent	gm.	per cent body weight	per cent	per cent	per cent
1	1	772	7.45 a.m.	134	35.7	4.62		1.94	71.7
	2	980	8.15 "	139	39.0	3.98		2.62	71.4
	3	747	8.30 "	118	26.4	3.53		2.40	75.4
	4	895	2.45 p.m.	118	31.3	3.50		2.52	72.2
	5	915	3.00 "	116	32.5	3.55		2.49	71.8
	6	780	3.15 "	114	24.7	3.17		3.03	71.5
	7	880	8.45 a.m.	116	25.2	2.86		2.35	70.9
	8	965	9.00 "	110	34.5	3.58		2.66	70.6
	9	940	9.15 "	122	31.9	3.39		2.79	71.1
	10	670	2.45 p.m.	113	19.3	2.88		3.10	71.8
	11	620	3.00 "	129	21.0	3.39		2.80	72.2
	12	958	3.15 "	130	30.5	3.18		2.86	71.8
Average.		844		122		3.47		2.63	71.9
2	13	525	8.40 a.m.	120	21.7	4.13	6.25	2.44	70.5
	14	570	9.00 "	145	27.4	4.81	7.66	2.69	70.8
	15	620	9.20 "	136	21.5	3.47	3.96	3.10	71.7
	16	573	2.40 p.m.	137	25.2	4.42	5.38	2.62	71.1
	17	500	3.00 "	136	20.7	4.14	4.73	2.84	71.7
	18	610	3.20 "	124	26.7	4.38	5.26	2.59	70.3
	19	610	8.40 a.m.	142	28.6	4.69	7.13	2.32	71.4
	20	650	9.00 "	139	33.2	5.11	6.95	2.33	71.9
	21	575	9.20 "	132	20.8	3.62	6.85*	2.54*	70.9
	22	560	2.40 p.m.	116	22.2	3.96	3.96	2.78	73.1
	23	645	3.00 "	139	34.4	5.33	7.83	2.30	71.5
	24	702	3.20 "	165	41.4	5.90	7.98*	2.40*	71.3
Average.		595		136		4.50	6.16	2.58	71.4
3	25	440	8.40 a.m.	137	19.7	4.48	7.75	2.57	72.2
	26	425	9.00 "	161	17.2	4.05	6.61	2.43	71.7
	27	400	9.20 "	154	14.9	3.73	5.45*	2.85*	71.1
	28	420	2.40 p.m.	129	17.1	4.07	7.48	2.41	71.5
	29	400	3.00 "	145	15.3	3.83	6.50	2.57	72.9
	30	370	3.20 "	123	14.3	3.86	3.78*	3.16*	72.4
	31	445	8.40 a.m.	167	21.8	4.90	9.46	2.20	71.9
	32	460	9.00 "	128	17.4	3.78	4.22	2.62	73.2
	33	375	9.20 "	133	15.7	4.19	7.59*	2.39*	71.7
	34	455	2.40 p.m.	190	20.3	4.46	9.04	2.44	71.3
	35	435	3.00 "	165	17.0	3.91	6.88	2.59	72.1
	36	370	3.20 "	184	18.3	4.95	9.05*	2.07*	72.2
Average.		416		151		4.18	6.98	2.53	72.0

\* Average of determinations on two different samples; see text.

We had reason to suspect several of the glycogen analyses in Series 1, and so have rejected all of these analyses for this series. Liver fat (total lipid) is expressed as per cent of oleic acid. Liver water content is expressed as per cent of fresh tissue, without correction for either fat or blood content. The water content exhibits only slight variations. In Series 2 and 3, for which both water and glycogen content data are available, the water content varies from 70.3 to 73.2 per cent, the glycogen from 3.78 to 9.46 per cent. This relative constancy of the water content, in association with wide variations in glycogen content, is in agreement with the recent studies of Fenn (1939) and other workers.

TABLE II  
*Differential Fat Analyses on Guinea Pig Liver Fat*

Guinea pig No.	Total lipid	Fatty acids (calculated)	Total cholesterol	Phospholipid P
	<i>per cent oleic acid</i>	<i>per cent oleic acid</i>	<i>mg. per 100 gm. liver</i>	<i>mg. per 100 gm. liver</i>
1	1.94	1.76	165	108
11	2.80	2.61	170	149
13	2.44	2.25	159	142
17	2.84	2.64	189	144
19	2.32	2.16	155	129
27 (Sample B)	2.85	2.66	188	171
30 ( " A)	3.14	2.90	216	174
34	2.44	2.28	141	146

In six of the animals glycogen and fat analyses were carried out upon samples taken from two different lobes of the liver (see "Material and methods"). The results were as follows: Guinea Pig 21, glycogen 6.62 (Sample A), 7.07 (Sample B), fat 2.35 (Sample A), 2.73 (Sample B); Guinea Pig 24, glycogen 7.80, 8.16, fat 2.25, 2.54; Guinea Pig 27, glycogen 5.46, 5.43, fat 2.84, 2.85; Guinea Pig 30, glycogen 4.01, 3.55, fat 3.14, 3.17; Guinea Pig 33, glycogen 7.74, 7.44, fat 2.37, 2.41; Guinea Pig 36, glycogen 9.06, 9.03, fat 1.86, 2.27. To summarize the above, the glycogen analyses were essentially identical in two of the animals, and differed by from 4 to 13 per cent in the other four; the fat analyses were essentially identical in three of the animals, and differed by from 13 to 22 per cent in the other three.



The total fat content of the liver was subjected to differential analysis for eight of the animals, representing all three series (Table II). The fatty acids comprise from 90.7 to 93.4 per cent of the total lipid, average 92.7 per cent. The phospholipid fatty acids may be calculated roughly by multiplying the phospholipid phosphorus (mg. of P per 100 gm. of liver) by 25 and taking two-thirds of the resultant figure. When these calculations are made for the data in Table II, the figures for the phospholipid fatty acids approach, equal, or somewhat exceed the figures for total fatty acids, and justify the conclusion that only a small portion of the fatty acids in the normal guinea pig liver is present as neutral fat.

As noted in the introduction, we sacrificed half of our animals (*i.e.*, half of each series) about 9 a.m. and half of them about 3 p.m. in order to check upon Petré's (1939) finding of a morning minimum and afternoon maximum in the liver glycogen content of the guinea pig. When the data upon the animals sacrificed in the morning and in the afternoon are summarized, it is at once apparent that the two groups of animals show no significant or consistent differences, either on the basis of liver glycogen content or on the basis of any of the other factors involved. Furthermore, the data are such as to make it almost certain that confirmation of Petré's finding would not be obtained simply by increasing the number of animals studied at the two sacrifice periods. The logical procedure would be to investigate a sufficiently large number of animals sacrificed at short intervals throughout the 24 hours. Such an extensive investigation we are not prepared to carry out at the present time. For a discussion of the problem of daily rhythmic variations in mammals, the reader is referred to (among others) the recent articles of Deuel *et al.* (1938), Sjögren *et al.* (1938), and Petré (1939), and to the monograph of Holmgren (1936).

The guinea pigs in Series 2 and 3 were weighed upon their arrival from the breeding farm in order to obtain some idea of the rate at which they gained weight under laboratory conditions when all elements of the diet were available in excess at all times. Series 2, maintained for 6 weeks, gained weight at the rate of from 4.5 to 7.9 gm. per day, average 5.9; Series 3, maintained for 3 weeks, gained weight at the rate of from 4.8 to 7.2 gm. per day, average 5.9.

## SUMMARY

Systematic data are presented upon the normal values of glycogen, fat, and water content of the livers of well nourished male guinea pigs. Animals sacrificed in the morning (9 a.m.) and afternoon (3 p.m.) show no significant differences on the basis of any of the factors investigated.

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# THE METAPHOSPHORIC ACID-PROTEIN REACTION\*

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Metaphosphoric acid is a strong precipitating agent for water-soluble proteins. This property, analytically, serves to distinguish it from the other phosphoric acids. The acid bound by the protein is carried down with the precipitate and is thereby separated from the acid left unbound. Schofield (1) has suggested that this property be utilized in the determination of the acid-binding capacity of proteins and Samuel and Schofield (2) have used such a method for estimating the acid-binding capacity of the proteins of wheat flour.

Herrmann and Perlmann (3) have reported that the maximum number of phosphorus atoms thus bound by an egg albumin molecule is very nearly equal to the number of positively charged groups ascertained to be present in the protein molecule by other methods. They state (4) that, as egg albumin is precipitated by metaphosphoric acid, both the amount of precipitate and the phosphorus content of the precipitate increase to maxima as increasing amounts of metaphosphoric acid are added to a given amount of protein.

It is the purpose of this paper to record some observations on metaphosphoric acid and its protein combination complex which are of interest in relation to this apparently stoichiometric reaction of metaphosphoric acid with proteins.

*Metaphosphate Reagent*—Samuel and Schofield (2) made titration curves on a number of commercially obtainable samples of

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glacial phosphoric acid and found two buffer ranges centering in the regions of pH 2 and 7. Metaphosphoric acid is known to be polymerized and they interpreted these buffer regions as evidence of the existence of at least two different types of polymers in the glacial acid. Variations in the buffer capacities in these two regions, from sample to sample, they interpreted as due to variations in the relative amounts of these two types of polymers present.

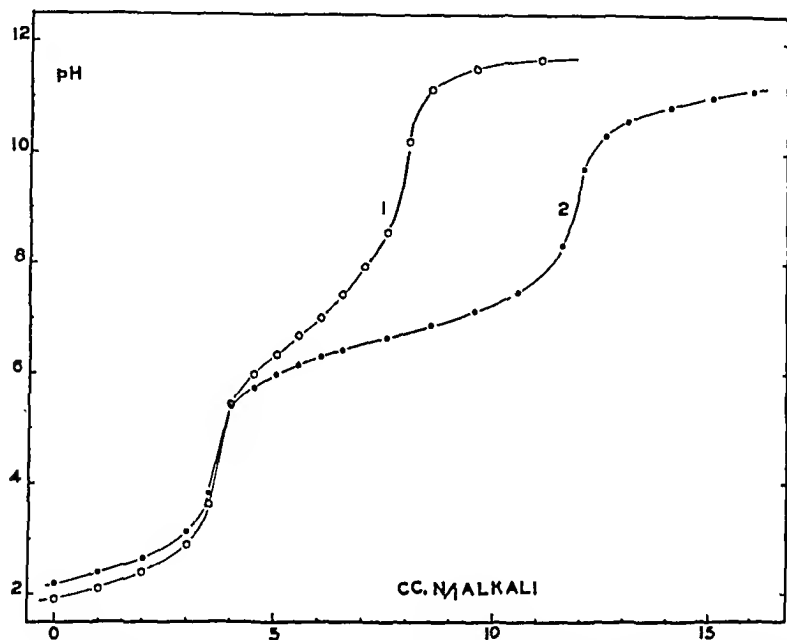


FIG. 1. Titration curves for 0.8 gm. of glacial metaphosphoric acid (Merck) in 100 cc. of solution before (Curve 1) and after (Curve 2) hydrolysis.  $N$  NaOH was used in the titration.

That a different explanation of these observations is possible is shown by the titration curves in Fig. 1. Curve 1 was made on a sample of freshly dissolved glacial metaphosphoric acid (Merck). 0.8 gm. of the glacial acid per 100 cc. of solution was titrated with  $N$  NaOH. Curve 2 was made upon an equal amount of the glacial acid after its solution had been boiled for 1 hour (volume kept constant). As is known, under this treatment the meta acid is hydrolyzed completely to the ortho acid. From these curves it is apparent (1) that the same acid (ortho) was being titrated in

both cases, (2) that base (some sodium must always be present in glacial metaphosphoric acid, otherwise the glass will not form) was present in an amount necessary to neutralize exactly one-half of the 1st hydrogen atom of the orthophosphoric acid present after complete hydrolysis of the sample, and (3) that one-half of the P in the glacial acid was originally present as orthophosphoric acid and one-half was present as metaphosphoric acid, apparently as the sodium salt (results were within 2 per cent of this proportion calculated on the basis of titration curves and the weight of the sample used). Titration of samples from other sources show that this almost exactly 1:1 ratio of ortho and meta acids is seldom encountered; hence, the differences in the buffer capacities of the samples in the two ranges.

The hydrolysis of metaphosphoric acid to orthophosphoric acid is markedly catalyzed by hydrogen ions. Because of variation in the meta acid content of commercial preparations and because, upon standing at laboratory temperatures, solutions of these preparations, being acidic, slowly lose their meta acid content (and, therefore, their property of causing precipitation of soluble protein), it has been suggested (5) that improvements in the usefulness of this reagent can be obtained if the metaphosphoric acid is prepared as a slightly alkaline solution of its sodium salt.

Sodium metaphosphate of negligible ortho- or pyrophosphate content is readily prepared by heating  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  to a temperature beyond the fusion point ( $616^\circ$ ) for a short period of time (30 minutes at  $750^\circ$  cause no detectable decomposition of the Na metaphosphate). The glass formed upon cooling is slowly soluble in water and its solutions give none of the qualitative tests for pyro- or orthophosphoric acid. Conductivity data obtained at various dilutions in water solution indicate a high degree of polymerization to be characteristic of this material. It is possibly the hexametaphosphate  $(\text{NaPO}_3)_6$  described in the literature.

Because the commercial metaphosphoric acid is found to be a mixture (with orthophosphoric acid) of uncertain composition, this "glass" form of sodium metaphosphate has been chosen as the metaphosphate reagent in these experiments. The pH of an aqueous solution of sodium metaphosphate (0.1 N) was found

to be the same as that of the  $\text{NaH}_2\text{PO}_4$  from which it was prepared; *i.e.*, pH 5.5. A titration curve made with a sample of the metaphosphate, however, showed that the buffer range of the 2nd and 3rd hydrogen atoms of the ortho acid had totally disappeared, while the buffer range of the 1 hydrogen atom of the

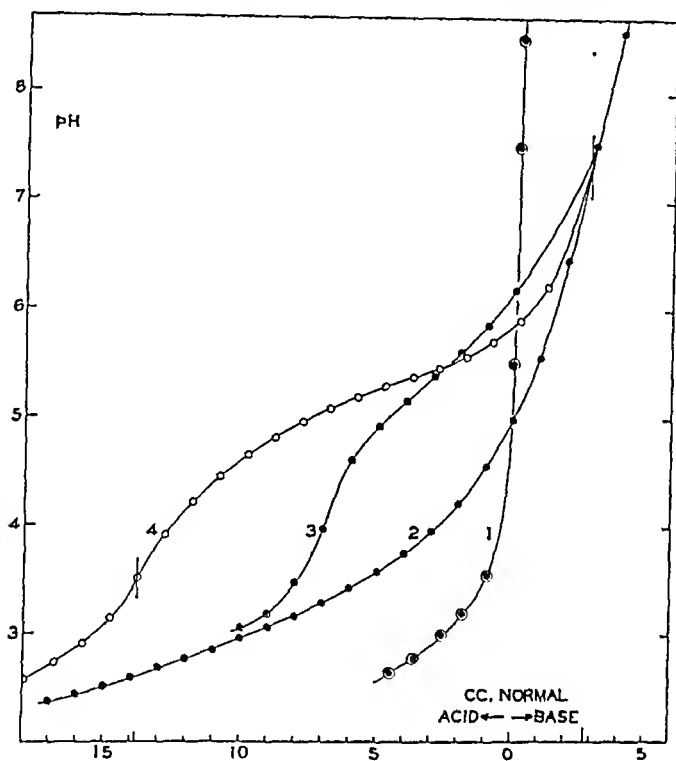


FIG. 2. Titration curves of (Curve 1) a solution containing  $2 \times 10^{-3}$  equivalent of sodium metaphosphate in 100 cc. of water, (Curve 2) a solution containing 1 gm. of serum albumin in 100 cc. of water, (Curve 3) a solution containing 1 gm. of serum albumin plus  $0.7 \times 10^{-3}$  equivalent of sodium metaphosphate in 100 cc. of water, and (Curve 4) a solution containing 1 gm. of serum albumin plus  $2 \times 10^{-3}$  equivalent of sodium metaphosphate in 100 cc. of water.  $N$  NaOH or  $N$  HCl was used in the titrations.

metaphosphoric acid approximated closely that of the 1st hydrogen atom of orthophosphoric acid (Curve 1, Fig. 2).

*Protein Reagent*—The protein used in these experiments was cow serum albumin, precipitated (after removal of the globulins) by saturated  $(\text{NH}_4)_2\text{SO}_4$ , dialyzed, and electrodialed free of

electrolytes. Its isoelectric point as shown by the pH of its completely electrodialed solution, and confirmed by cataphoresis measurements, was at pH 4.98. Curve 2, Fig. 2, is the titration curve for a solution of this protein.

### *Protein-Metaphosphate Reaction*

When small increments of a 0.1 N solution of Na metaphosphate (brought to pH 5.0 by addition of a trace of HCl) are added to a 1 per cent solution of the protein, the solution remains clear and it is found that the pH changes toward the alkaline side in a manner shown in Fig. 3; that is, there is an abrupt rise in pH to about 6.2 upon addition of the first small increments of metaphosphate, followed by a slow drop in pH until no further change occurs after addition of 20 cc. of the 0.1 N solution (2 milliequivalents of  $(\text{NaPO}_3)_2$  per gm. of protein). If, now, this clear solution is titrated with HCl, it is found that the titration curve of the mixture follows a course quite different from that of the original protein (Curve 4, Fig. 2), and that the solution becomes cloudy at a pH of 5.2, followed by a heavy precipitation of the protein as the pH is decreased. Addition of greater amounts of the sodium metaphosphate causes no appreciable change in the course of this titration curve. If, however, a titration curve is followed through when only 7 cc. of 0.1 N metaphosphate (0.7 milliequivalent of  $(\text{NaPO}_3)_2$  per gm. of protein) have been added, it is found to follow the course given in Curve 3, Fig. 2.

The titration curve of the protein-metaphosphate is reversible. No denaturation of the protein occurs through the action of the metaphosphate, as is evidenced by the fact that, when the solution containing protein and metaphosphate is dialyzed at  $\text{pH} > 7$ , the metaphosphate is readily removed and the protein is obtained with all of its original properties unchanged. When dialysis is carried out at  $\text{pH} < 4.5$ , however, it is not possible to remove the metaphosphate quantitatively. Electrodialysis under these conditions leads to a strong denaturation of the protein.

The titration curves in Fig. 2 show the following relationships. (a) When protein and metaphosphate are allowed to react, there appears a pronounced buffer segment in the titration curve of the mixture located in a pH range in which neither of the reagents alone shows any marked buffer capacity. The mid-



point of this buffer segment of the titration curve of the mixture is at pH 5.2 and extends from pH 3.5 to 7.0, coinciding at the latter point with the point of inflection on the titration curve of the original protein (in the absence of metaphosphate). (b) This new buffer segment becomes increasingly pronounced as the amount of metaphosphate in the mixture is increased, until a maximum is attained, beyond which further addition of metaphosphate has little or no effect upon the course or extent of the buffer curve in this region of pH. The amount of acid or base required to titrate this buffer segment of the titration curve is found to be approximately  $166 \times 10^{-5}$  equivalent per gm. of the protein when the metaphosphate content of the mixture is greater than  $200 \times 10^{-5}$  equivalent per gm. of protein. When much less metaphosphate than this amount is present, this buffer segment is of a correspondingly lower extent (Curve 3, Fig. 2).

It may be concluded from these data that the reaction of metaphosphate with protein is of the double decomposition type, involving the ionizable basic groups on the protein through the formation of a protein metaphosphate with extremely low dissociation tendency. A low but definite excess of metaphosphate is required for the maximum development of this masking effect of the basic groups of the protein. The displacement of the pH range in which the acidic groups of the protein can be titrated when the basic groups are thus bound by the metaphosphate may be explained upon the assumption that, when the basic groups are masked, the dissociation constant of the acidic groups becomes lower. A parallel observation on the shift in the dissociation constant of the basic group of glycine is encountered when the carboxyl group is esterified (6).

On the basis of this explanation of the change in the dissociation constant of the acidic groups of the protein, the conclusion would be drawn that the buffer segment, centered at pH 5.2, represents the titration of the  $\text{—COOH}$  groups of the protein. In terms of the zwitter ion theory, it is a measure of the acid-binding capacity of the protein. The equivalents of base or acid required to titrate this buffer segment,  $166 \times 10^{-5}$  equivalent, approximate fairly closely the acid-binding capacity of serum albumin. Pauli and Valkó (7) state the acid-binding capacity of serum albumin to be  $155 \times 10^{-5}$  equivalent per gm. of

protein (the average of several reported values ranging from  $147 \times 10^{-5}$  to  $167 \times 10^{-5}$  equivalent).

Such an explanation of the shift in the dissociation constant of the acid groups of the protein would necessarily imply a very strong bonding between the basic groups of the protein and the metaphosphate ions. It would be expected that a similar effect would be observable in the case of an amino acid. It is found, however, that the titration of glycine in the presence of sodium metaphosphate shows no such displacement of the titration range of the  $-\text{COOH}$  groups of the amino acid. Titration curves of other proteins, *e.g.* egg albumin and casein, in the presence of

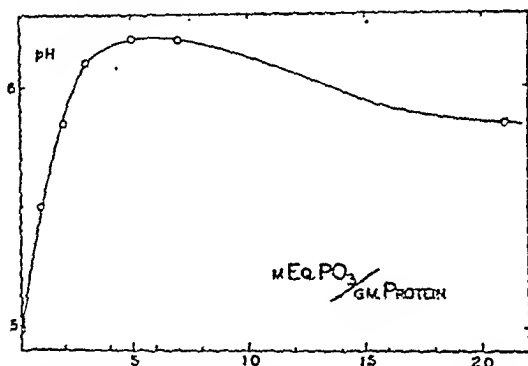


FIG. 3. Change in pH of a salt-free 1 per cent serum albumin solution (isoelectric point, pH 4.98) when increments of sodium metaphosphate (adjusted to pH 5.0) are added.

$(\text{NaPO}_3)_x$  all show similar buffer segments not present in the titration curve of the protein or the metaphosphate alone. The extent of such buffer segments in the cases of all proteins studied corresponds very closely to the accepted acid-binding capacities of the proteins. The whole phenomena of metaphosphate-binding by proteins seems to be intimately associated with the polyvalent character of the protein and of the metaphosphate (polymerized) rather than some specific attraction between amino groups and the metaphosphate ion. Nevertheless, the amino groups are involved in the reaction, since they are so strongly masked in the complex.

That the protein-metaphosphate complex has many of the char-

acteristics of a complex coacervate (or flocculate), as studied and defined by de Jong (8), is indicated by the observation of Koets.<sup>1</sup> Polyvalent electrolytes commonly have the property of causing a phase separation (as coacervate or flocculate) when added in relatively small amounts to solutions of lyophilic colloids bearing a charge opposite to that of the polyvalent ion of the electrolyte. That the metaphosphate exists as an electrolyte with a high valent anion, as a result of its polymerizing tendency, is evident from the conductivity data mentioned above.

A characteristic of coacervation in general is the dissolving action of added neutral salts. Perlmann and Herrmann (4) observed this action of neutral salts upon the metaphosphate egg albumin precipitate. Koets found the same effect in the case of serum albumin precipitated by a minimal amount of metaphosphoric acid. With higher concentrations of metaphosphate, reversal of the precipitation by neutral salts was not possible, however.

De Jong and van der Linde (9) have found that with various polyvalent ions of charge opposite to that of a colloid electrolyte the amounts of such ions bound by unit weight of the colloid, when the electrokinetic potential of the resulting coacervate was reduced to zero value, were equivalent (pH held constant). Also, the number of equivalents of the polyvalent ion required in such cases bore a nearly constant ratio to, and was consistently greater than, the number of equivalents of replaceable ions present per unit weight of the colloid. Increasing the concentration of the polyvalent salt results in a further binding of the polyvalent ion within the complex. A reversal of charge of the colloid phase then results if the pH is held constant. There is always a definite amount of the polyvalent salt (left) in the equilibrium liquid in contact with the precipitated phase. While it is probable in all such cases that primary electrovalences are involved in the coacervate formation, the relationship between the amount of polyvalent crystalloid ion bound per unit weight of colloid and the amount of polyvalent ion remaining in the equilibrium solution is described by an adsorption isotherm.

When metaphosphate is added to a protein, it is found that the

<sup>1</sup> Koets, P., private communication.

isoelectric point of the colloid phase is shifted markedly toward the acid range of the isoelectric point of the original protein. The extent of this shift is a function of the amount of metaphosphate present per unit amount of protein. If the amount of metaphosphate which is required to shift the isoelectric point of the protein-metaphosphate complex to any chosen pH value is determined for varying concentrations of serum albumin and for vari-

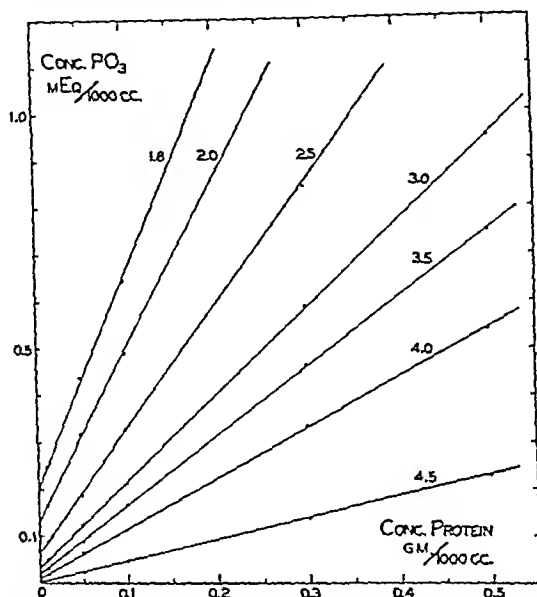


FIG. 4. Total metaphosphate needed to bring the isoelectric point of the protein-metaphosphate complex to the indicated values of pH as the total concentration of protein is varied.

ous pH values, a set of curves is obtained as shown in Fig. 4. It is found that, with varying protein concentrations maintained at a given pH, the amounts of total added metaphosphate required to bring the cataphoretic motion of the precipitated particles to zero all lie on a straight line which cuts the axis (corresponding to metaphosphate concentration) at a point above the origin. As the pH (isoelectric) is made more acid, the slope of the line and the point at which it cuts this axis both increase. The slope of the straight line obtained is proportional to the

equivalents of metaphosphate bound per unit weight of protein and the intercept on the metaphosphate-concentration axis is a measure of the equivalents per liter of metaphosphate present in the equilibrium liquid.

By plotting the equilibrium concentration ( $C$ ) of metaphosphate against the amount bound by unit weight of protein ( $X$ ), a Freundlich adsorption isotherm is obtained. The data do not fit the Langmuir adsorption isotherm. The log-log curve is a straight line with a slope closely approximating a value of 0.5, which indicates that the amount of  $\text{PO}_3$  bound by unit weight of the protein varies as the square root of the equilibrium concentration; i.e.,  $X = K\sqrt{C}$ . This is not in accord with the conclusion of Perlmann and Herrmann that a maximum in the  $\text{PO}_3$

TABLE I

*Amount of  $\text{PO}_3$  Bound per Gm. of Serum Albumin and Concentration of  $\text{PO}_3$  in Equilibrium Liquid Which Will Bring the Protein-Metaphosphate Complex to Isoelectric Reaction at Various pH Values*

Read from Fig. 4.

pH (isoelectric).....	4.5	4.0	3.5	3.0	2.5	2.0	1.5
Equilibrium concentration of $\text{PO}_3$ , m.eq. per 1000 cc..	0.002	0.010	0.020	0.032	0.064	0.132	0.204
$\text{PO}_3$ bound, m.eq. per gm. protein.....	0.46	1.07	1.46	1.84	2.64	3.65	4.51

bound is reached when the  $\text{PO}_3:\text{NH}_2$  ratio is 1. Table I gives the numerical values of the equilibrium concentration of  $\text{PO}_3$  and the amounts of  $\text{PO}_3$  bound by 1 gm. of protein as obtained graphically from Fig. 4.

#### DISCUSSION

From that which is so far known of the metaphosphate-protein reaction, it can best be regarded as a complex in which the negative multivalent (polymerized) metaphosphate ion is linked to the positive (amino) groups of the protein by a salt-like bond of very low ionizing capacity.

When neutral sodium metaphosphate is added to isoelectric salt-free protein, the first increments are bound almost completely, the equilibrium concentration of metaphosphate in solution being almost negligibly small. Because of the low ionizing

(dissociation) tendency of the protein-metaphosphate, a shift in the isoelectric point of the complex toward the acid range takes place. As the amount of added Na metaphosphate is increased, the amount bound by the protein increases, following an adsorption isotherm, and the isoelectric point is shifted further toward the acid range (the basic groups being strongly masked by the metaphosphate). The fraction of the positive ions of the polyvalent metaphosphate polymer which is replaced by the amino groups of the protein decreases as the total metaphosphate present increases so that the ratio,  $\text{PO}_3:\text{NH}_2$ , increases continuously with increase in metaphosphate.

In the case of serum albumin, by the time the equivalent concentration of metaphosphate added has reached  $2 \times 10^{-2}$  per gm. of protein (in 100 cc. of water), the number of amino groups combined with (masked by) metaphosphate has reached close to a maximum. More metaphosphate added still leads to an added amount of metaphosphate bound but this increase results from a lower number of protein (*i.e.* amino) groups binding each molecule of metaphosphate polymer. A limit should theoretically be reached when one metaphosphate polymer is combined with each amino group in the protein.<sup>2</sup>

The strong masking of the amino groups of the protein by the metaphosphate apparently leads to a change in the dissociation constant of the carboxyl groups of the protein and to the appearance of a buffer segment in the titration curve of the complex which is not present in the titration curve of either component alone. The extent of this new buffer segment is very nearly equal to (slightly greater than) the accepted acid-binding capacity of the protein and probably represents the titration range of the acidic groups of the protein (though in a different pH range from that which is characteristic of the titration of the acid groups of the original protein on the basis of the zwitter ion theory).

#### SUMMARY

The  $\text{PO}_3$  content of the protein-metaphosphate complex depends upon the  $\text{PO}_3$  concentration of the equilibrium liquid in a manner described by the adsorption isotherm. A salt-like, low

<sup>2</sup> On the basis of this theory we would expect the metaphosphate binding by the protein to follow the Langmuir isotherm. Why it does not do so exactly but does follow the Freundlich isotherm cannot as yet be explained.

ionizing combination between the polyvalent metaphosphate ion and the basic groups of the protein is, however, clearly involved.

Formation of this complex leads to a masking of the  $\text{—NH}_2$  groups of the protein and a shift in the dissociation constant of the  $\text{—COOH}$  groups to a pH region in which they may be readily titrated. As the metaphosphate concentration is increased relative to that of the protein, this buffer segment of the titration curve of the protein-metaphosphate complex reaches a maximum value which approximates closely the acid-binding capacity of the protein, while the amount of metaphosphate bound by the protein continues to increase in accord with the adsorption isotherm.

That the phenomenon is of the nature of a complex flocculation is indicated by many properties of the complex. No similar reaction occurs between metaphosphate and amino acids or other low molecular weight substances containing single basic groups.

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# PHOTOCHEMISTRY AND ABSORPTION SPECTROSCOPY OF THE PYRIMIDINE COMPONENT OF VITAMIN B<sub>1</sub>

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(Received for publication, March 2, 1940)

The absorption spectrum of vitamin B<sub>1</sub> (thiamine) has been determined by several investigators. It has been found by some (1) that two absorption bands are present with maxima at 2350 and 2670 Å. respectively, whether the solvent is alcohol or water, but others have reported only a single absorption band around 2450 to 2470 Å. It is now clear that this lack of agreement is due to the fact that the absorption spectrum of thiamine is a function of the hydrogen ion concentration, the two maxima appearing for a pH of 7 or greater and only one at a pH of 5.5 or less (2). That the absorption behavior of thiamine may be attributed to its pyrimidine component is suggested by recent studies (3) on a number of 6-aminopyrimidines in acid and alkaline media, in which there was observed a similar change of absorption; the thiazole portion of the thiamine molecule exhibits a single absorption band around 2530 Å. (4). Holiday (2) found thiamine to obey Beer's law for the narrow range of concentrations investigated, within the limits of error of his measurements ( $\pm 2$  per cent).

In the present study, the variation in absorption with pH for the pyrimidine component of thiamine has been measured as a prelude to the subsequent photochemical investigation. A sample of 2-methyl-5-ethoxymethyl-6-aminopyrimidine, obtained from Merck and Company through the courtesy of George W. Lewis, and referred to hereafter as simply "pyrimidine," has been used for all the reported measurements. The data have been obtained with a medium Hilger spectrograph and Spekker photometer, a tungsten steel spark source, Eastman No. 33 plates, and 1 cm.



absorption cells. The concentration employed was  $6.25 \times 10^{-5}$  M. At pH 4.8, 5.9, 6.4, and 7.0, phosphate buffer solutions were used, the pH values being measured with a glass electrode. For the curves labeled pH 1.5 and 13.0, we used a  $M/15$   $H_3PO_4$  solution and a 2 per cent solution of KOH, respectively.

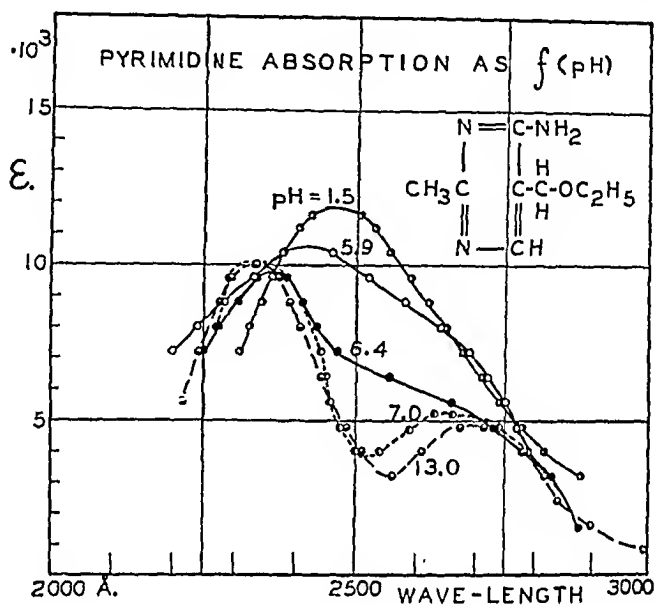


FIG. 1. The absorption spectrum of 2-methyl-5-ethoxymethyl-6-aminopyrimidine as a function of pH.

The results are shown in Fig. 1 for the wave-length range 2200 to 3000 Å., where the value of the molecular extinction coefficient ( $\epsilon$ ) is plotted as the ordinate.  $\epsilon$  is defined by the equation

$$\epsilon = \frac{1}{cd} \log_{10} \left( \frac{I_0}{I} \right)$$

where  $c$  is the concentration in gm. moles per liter,  $d$  is the path length in cm., and  $I_0$  and  $I$  are the incident and transmitted intensities, respectively. It will be seen that two absorption bands are present at a pH of 7.0 or more, while only one is present at a pH of 1.5. The region of most rapid change in absorption with pH lies between the values 5.5 and 7.0, in agreement with the finding for thiamine (2). Because solutions in distilled water may readily undergo changes in pH owing to  $CO_2$  uptake

from the atmosphere, it was essential to buffer solutions in order to maintain known and constant absorption characteristics. Similarly, as subjecting solutions of pyrimidine to radiation might alter the hydrogen ion concentration, the precaution of using a buffer to keep the absorption behavior constant for the unchanged pyrimidine seemed doubly advisable. Beer's law was found to be obeyed within the limits of error of the measurements for the concentrations upon which the photochemical yields are based.

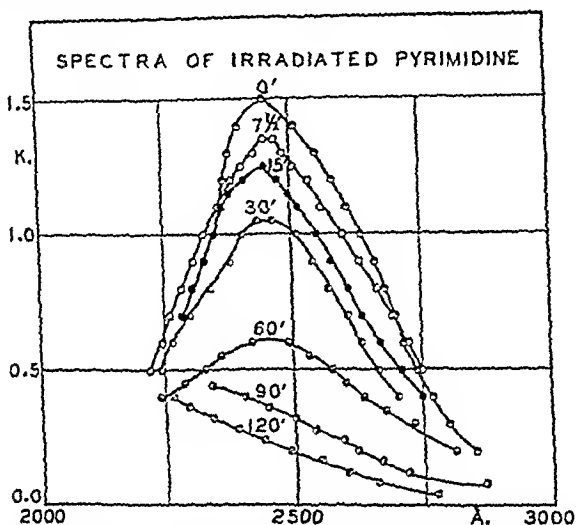


Fig. 2. Absorption spectra of irradiated thiamine "pyrimidine." See Table I for values of absorbed energy.

During ultraviolet irradiation of a  $12.5 \times 10^{-3} M$  solution of the 2-methyl-5-ethoxymethyl-6-aminopyrimidine, its absorption spectrum undergoes the changes shown in Fig. 2, where the extinction coefficient,  $k$ , represents the product of  $\epsilon$  and  $c$ . The radiation was furnished by a low pressure mercury discharge tube (Hanovia Sc-2537) emitting almost solely the  $\lambda$  2537 Å. line of mercury. The energy incident on the solution for the various time intervals indicated on the curves is given in ergs per cc. in Column 2 of Table I. A steady decline in the absorption maximum can be noted until finally all selective absorption disappears. This signifies the decomposition of the unsaturated pyrimidine

ring structure, since the presence of double bonds in the ring always results in selective absorption in the range 2400 to 2700 Å. Just what the breakdown products are is not known, with the exception of ammonia nitrogen. A test for ammonia by nesslerization gave a yield which could be accounted for by about one-third of the amino nitrogen present in the molecule being split off and suggested that the amino and possibly other side groups were frequently split off before actual decomposition of the ring structure itself. A convenient and sensitive reaction for testing this hypothesis is available in one of the biological assay methods for thiamine and will now be described.

TABLE I  
*Absorption Data for Irradiated "Pyrimidine"*

Exposure time	Incident energy	$k(2537 \text{ Å.})$ for solution	$k^1(2537 \text{ Å.})$ for active "pyrimidine"	$A/I_0$ for solution	$A^1/I_0$ for active "pyrimidine"
(1)	(2)	(3)	(4)	(5)	(6)
min.	ergs per cc.			per cent	per cent
0	0.0	1.33	1.33	95.3	95.3
7.5	$0.763 \times 10^7$	1.19		93.4	
15	$1.526 \times 10^7$	1.04	0.70	90.9	80.0
30	$3.052 \times 10^7$	0.90	0.39	87.4	59.2
60	$6.152 \times 10^7$	0.54	0.11	71.2	22.5
90	$9.261 \times 10^7$	0.29	0.02	48.7	4.7
120	$12.364 \times 10^7$	0.17	0.00	32.4	0.0

It has been shown (5, 6) that the fungus, *Phycomyces blakesleeanus*, can develop quite as efficiently when administered equimolecular quantities of the two components of vitamin B<sub>1</sub> as when given the thiamine itself. The dry weight of the cultures was found to be a function of the concentration, so that a method of assaying thiamine can be based on the growth characteristics of the fungus. Similarly, an assay for the concentration of either thiamine component can be carried out when the concentration of the other is known. In the case of the thiamine "pyrimidine," inactivation may mean only the removal of either the 6-amino group or the 5-ethoxy group from the ring rather than a breakdown of the ring itself, as each of the two groups mentioned has been shown to be essential for growth (6, 7). Since

the decomposition of the ring itself is reflected in the loss of selective absorption, these two types of photochemical inactivation may be distinguished experimentally.

#### EXPERIMENTAL

In our investigation, each liter of nutrient solution contained 100 gm. of dextrose (cerelose), 4.0 gm. of *l*-asparagine, 1.5 gm. of  $\text{KH}_2\text{PO}_4$ , and 0.5 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in redistilled water, together with 4-methyl-5- $\beta$ -hydroxyethylthiazole to give a concentration of  $0.5 \times 10^{-7}$  M. This concentration of the thiamine "thiazole" was maintained constant for all determinations of the thiamine "pyrimidine." The latter was prepared as a stock solution at  $12.5 \times 10^{-5}$  M, buffered at pH 4.8 in a M/15 solution of  $\text{KH}_2\text{PO}_4$ , and the irradiation exposures were made at this concentration. For the control growth curves, the "pyrimidine" concentration in the nutrient medium was varied from  $0.5 \times 10^{-7}$  M down to zero. After 25 cc. of nutrient solution were added to each of ten 125 cc. Erlenmeyer flasks and the contents sterilized for 12 minutes at 15 pounds pressure, each flask was inoculated with 2 drops of a sterile spore suspension from cultures of *Phycomyces blakesleeanus*, plus strain, which had been growing for 10 or more days on potato-dextrose-agar slants. The mature cultures in the flasks, having grown for 10 days in a dark oven at 23–24°, were autoclaved and the mycelial mats removed, washed, dried for 24 hours at 95–97°, and weighed.

The control growth curves showing the average dry weight in mg. per culture flask as a function of the "pyrimidine" concentration for two different determinations are shown in Fig. 3. In the concentration range  $0.0125$  to  $0.150 \times 10^{-7}$  M, the dry weight is seen to be roughly proportional to the concentration. At still higher concentrations of "pyrimidine," the growth became limited by the "thiazole," whose concentration was maintained constant at  $0.5 \times 10^{-7}$  M. The rather high dry weight when no "pyrimidine" was added was apparently due to slight traces of it present as an impurity in the dextrose or the asparagine; if no "thiazole" were added either, then the dry weight amounted to only 0.4 mg. The asparagine was recrystallized twice from redistilled water.

For the photochemical inactivation of the "pyrimidine," a

cylindrical fused quartz irradiation cell with plane-parallel faces was filled with approximately 5 cc. of a  $12.5 \times 10^{-5}$  M solution, buffered at pH 4.8, and placed at a distance of 5.8 or 7.0 cm. from the low pressure mercury discharge tube, which operated on a sign transformer rated at 5000 volts, 100 milliamperes. The cell had an opening through which a motor-driven stirrer functioned continuously during irradiation. The radiation was in-

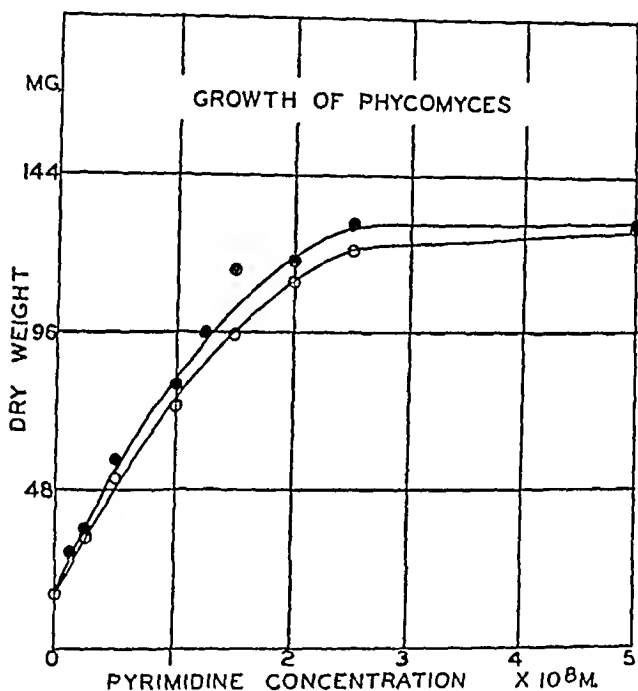


FIG. 3. Increase in dry weight of *Phycomyces* cultures as a function of "pyrimidine" concentration, with "thiazole" concentration constant (two separate trials).

cident on one of the two circular faces of the cell and its intensity was measured with a vacuum type thermopile. Since the thickness of the absorbing layer of solution was very nearly 1 cm. (1.0057 cm.), the energy incident per sq.cm. also represented the energy incident per cc. of solution.

At appropriate intervals during irradiation, a 0.05 cc. sample of the solution was withdrawn from the cell and its "pyrimidine" concentration determined by a *Phycomyces* assay based on the

culture procedure just outlined and on a comparison with the control growth curves. For this comparison, the growth for any given dose of radiation in the series was computed as a percentage of the growth by a non-irradiated control for a concentration of  $0.25 \times 10^{-7} \text{ M}$ , made during the same series; a similar calculation was made for the control series data plotted in Fig. 3, and the comparisons were then made on this percentage basis.

The validity of the *Phycomyces* assay for irradiated pyrimidine was tested by growing three series of ten cultures differing only in that Series A contained 0.05 cc. of  $12.5 \times 10^{-5} \text{ M}$  unirradiated pyrimidine, Series B contained 0.05 cc. of the same solution irradiated for several hours, and Series C contained 0.05 cc. of each of these solutions. The growth in Series C was approximately equal to the sum of the growth in Series A and B, indicating that the end-products of pyrimidine inactivation do not inhibit the growth of *Phycomyces*.

In order to calculate the radiant energy absorbed at  $\lambda 2537 \text{ \AA}$ . by the solution during an exposure interval, it was necessary to measure the absorption from time to time. This was done by interrupting the exposure and determining the absorption spectrum with the Hilger Spekker photometer, securing curves like those of Fig. 2. With the incident energy and the per cent absorption known as a function of time, the total energy absorbed per cc. during an interval could be calculated. The value determined in this way represents not only the energy absorbed by the active "pyrimidine," but also by the various possible inactivation products. Since the concentration ( $c$ ) of the active component is known from the *Phycomyces* assay, and its extinction coefficient is equal to  $\epsilon c$ , where  $\epsilon$  is the molecular extinction coefficient at  $\lambda 2537 \text{ \AA}$ . obtainable from the top curve of Fig. 2, it is possible to calculate that portion of the energy absorbed by the active "pyrimidine." The fraction in question is the ratio of the product ( $\epsilon c$ ) just mentioned to the value of  $k$  determined from the curve in Fig. 2 after a particular period of irradiation.

### Results

The data given in Table I represent the conditions of Fig. 2, but give in addition the total energy incident per cc., the per cent absorbed at  $2537 \text{ \AA}$ . by the solution ( $A/I_0$ ), and finally

the per cent of the incident energy per cc. which is absorbed by the active "pyrimidine" ( $A^1/I_0$ ) at the end of the time intervals indicated.

Upon plotting the extinction coefficients for the inactive decomposition products as a function of wave-length for various

TABLE II  
Quantum Yield Data for "Pyrimidine" Inactivation

Exposure time	Dry weight of cultures	Pyrimidine inactivated	Average energy absorbed per interval	Total energy absorbed by active pyrimidine in		Quantum yield
				Joules per cc.	Quanta per original molecule	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
min.	mg. per flask	per cent	per cent			
0	131.6 ± 1.7	0.0		0.00	0.0	
15	94.7 ± 1.6	53.0	70.7	1.34	22.8	0.0232
30	63.1 ± 1.0	75.5	45.5	2.15	36.5	0.0207
45	43.0 ± 1.0	87.7	27.7	2.64	44.9	0.0195
60	28.4 ± 0.8	95.8	17.5	2.95	50.2	0.0191
90	23.9 ± 0.4	98.0	10.7	3.14	53.4	0.0183
0	124.8 ± 4.0	0.0		0.00	0.0	
28	73.3 ± 1.0	65.0	65.9	2.16	36.7	0.0177
60	35.4 ± 1.3	94.0	25.3	3.10	51.8	0.0178
90	22.6 ± 0.8	99.4	9.1	3.42	58.0	0.0171
0	128.7 ± 3.0	0.0		0.00	0.0	
30	64.3 ± 2.1	72.5	64.6	2.30	39.2	0.0187
60	26.9 ± 2.1	96.0	23.9	3.14	58.0	0.0181
90	17.9 ± 0.9	100.0				
0	124.1 ± 1.3	0.0		0.00	0.0	
100	67.2 ± 0.7	70.0	64.2	2.38	40.5	0.0173
125	57.9 ± 0.6	76.8	29.8	2.66	45.2	0.0170
150	43.5 ± 0.7	85.8	23.4	2.88	48.9	0.0175
0	112.5 ± 1.4	0.0		0.00	0.0	
90	69.1 ± 0.7	63.4	65.6	2.18	37.0	0.0171
120	51.3 ± 0.9	77.5	32.1	2.53	43.0	0.0180
150	45.4 ± 1.4	82.0	24.0	2.79	47.4	0.0174
Average.....						0.0184

times of irradiation, it was found that they exhibited selective characteristics. This indicates that inactivation has resulted from the splitting off of radicals from the 2-methyl-5-ethoxymethyl-6-aminopyrimidine as well as from a breakdown of the ring structure. The more probable reaction would seem to be

the removal of the essential amino group rather than the ethoxymethyl group, since it is known that the C—N bond is much weaker than the C—O bond. That ammonia is a common decomposition product of irradiated amino acids (8) gives some indication of its probability of detachment from organic molecules.

Quantum yields for the inactivation of the essential "pyrimidine" have been calculated from five different irradiation trials, a summary of which is given in Table II. The dry weight in each case is the average for ten flasks (in the fourth and fifth trials, for fifteen to thirty flasks) and the probable errors are given in each case. Column 4 represents the average in per cent of the incident energy absorbed by the active "pyrimidine" during the respective irradiation intervals, while Columns 5 and 6 give the total energy absorbed in joules per cc. for various irradiation times and the quanta absorbed per molecule of "pyrimidine" initially present, respectively. The last column shows the quantum yield at 2537 Å. as determined for each interval separately. The average value is seen to be 0.0184, which means that on the average only 1 molecule is inactivated for every 54 quanta absorbed.

Earlier studies (9, 10) on the effect of ultraviolet radiation upon substances containing the pyrimidine ring have been based entirely on changes in their absorption spectra. This has led in the case of nucleic acid to the conclusion that it is very stable, having suffered almost no change in its absorption spectrum after an hour's exposure to the full radiation of a mercury arc under conditions presumably yielding a greater ultraviolet energy than is found in the present work to be sufficient for completely inactivating the "pyrimidine." This conclusion seems unjustified, because the pyrimidine and purine constituents of nucleic acid, being held to the molecule through C—N linkages, may be split off and the solution would still continue to give the characteristic absorption spectrum of nucleic acid. This latter statement is based on the fact (11) that the molecular extinction coefficient of nucleic acid is equal to the sum of the coefficients of its constituents, at least approximately.

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## SUMMARY

The absorption spectrum of 2-methyl-5-ethoxymethyl-6-aminopyrimidine has been found to vary with pH in the same manner as that of thiamine (vitamin B<sub>1</sub>), and is evidently responsible for the behavior of the latter.

The photochemical decomposition of this thiamine "pyrimidine" at  $\lambda$  2537 Å. has been demonstrated by its loss of selective absorption and its inability to support the growth of *Phycomyces* cultures.

The quantum yield for inactivation, when inactivation results from changes in side groups as well as from a breakdown of the ring structure, has been found to be 0.0184.

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# THE EFFECT OF THE RATE OF ABSORPTION OF GLUCOSE ON THE PHOSPHATES IN THE INTESTINAL MUCOSA\*

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In an effort to test the validity of the hypothesis that phosphorylation is concerned with the selective absorption of certain hexoses, Verzář and Süllmann (1) studied the effect of the absorption of sodium chloride, glucose, fructose, galactose, mannose, and glycerol on the concentration of several fractions of the acid-soluble phosphate in the intestinal mucosa. Their data show that the concentration of the organic acid-soluble phosphate that accompanied the absorption of hexoses and of glycerol exceeded the concentration that was observed during the absorption of sodium chloride. However, since the order of the increases that were observed did not correspond with the order of the velocity of absorption of the respective hexoses, it was concluded that no statement could be made concerning the specificity of these changes. Such a comparison may have been affected by the occurrence of side reactions which involve phosphorylation and which may have differed in the case of the different sugars. Also, some of the hexoses employed form monophosphoric acid esters, while at least one (fructose) may form a diphosphoric acid ester. These considerations raise some doubt as to the significance of a comparison between the rates of absorption of *several* sugars and the concentration of the several fractions of the acid-soluble phosphate in the intestinal mucosa.

A recent study by Althausen and Stockholm (2) makes possible a different approach to this problem. These workers demonstrated that the administration of thyroxine to rats markedly

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increased the rate of absorption of substances susceptible to phosphorylation, such as glucose, galactose, and oleic acid, and that thyroidectomy decreased the rate of absorption of glucose. Accelerated emptying time of the stomach, increased basal metabolic rate, increased rate of blood flow, hyperperistalsis, and increased permeability of the mucosa were ruled out as factors accelerating the rate of absorption of glucose in the rat treated with thyroxine. It was suggested that thyroxine increases intestinal absorption through stimulation of the phosphorylation mechanism.

In this connection it was thought that it might be more significant to compare the concentrations of the several fractions of the acid-soluble phosphate in the intestinal mucosa during the absorption of a *single* hexose (glucose) under conditions of accelerated, normal, and retarded rates of absorption. A positive correlation between the concentration of phosphates in the mucosa and the rate of absorption would support the hypothesis that phosphorylation is a process concerned with the selective absorption of certain hexoses. A lack of correlation need not militate against such a hypothesis, since it is possible that the concentrations of the several fractions of phosphate are governed by a steady state that is not greatly influenced by changes in the rate of absorption.

#### EXPERIMENTAL

Female rats weighing between 180 and 200 gm. were employed throughout this investigation. In order to clear the intestine of food residues, the animals were fasted in a metabolic cage during a period of 48 hours.<sup>1</sup> They were then given, by means of stomach tube, 3 cc. of a 20 per cent solution of glucose. This amount of glucose is more than the animal is able to absorb during a period of 45 minutes. 3 cc. of an isotonic solution of sodium chloride were given to a control group of rats. 45 minutes after administration of glucose or sodium chloride the animals were sacrificed, the intestines were removed, and the mucosal surface was rapidly washed with two 5 cc. portions of a cold isotonic

<sup>1</sup> It was found necessary to administer a cube of sugar after the first 24 hours of fasting to prevent death in the thyroxinized animals. The normal and thyroidectomized animals received the same treatment.

solution of sodium chloride.<sup>2</sup> The mucosa was scraped from the intestine, frozen with solid carbon dioxide, and pulverized. All operations were conducted as rapidly as possible in order to prevent hydrolysis of phosphate esters.

A portion of the finely powdered mucosa was reserved for determination of the water content. Another portion (1 to 2 gm.) was weighed in a tared bottle that contained 24.0 cc. of a 10 per cent solution of trichloroacetic acid. The mixture was triturated for 15 minutes and filtered. Such a procedure yielded a clear, protein-free filtrate. The directly determined acid-soluble phosphate, the total acid-soluble phosphate, and, by difference, the organic acid-soluble phosphate were determined by the method of Fiske and Subbarow (3). In addition to inorganic phosphate, the directly determined phosphate contains about 5 per cent of phosphate that results from the hydrolysis of creatine phosphate (1). Lohmann's method (4) was used to characterize the organic acid-soluble phosphate. According to this method, the trichloroacetic acid filtrate is hydrolyzed by 1 N hydrochloric acid at 100° for varying lengths of time. The intervals of time that were employed in this work were 7, 30, and 180 minutes. The concentration of phosphate that was liberated by 180 minutes of acid hydrolysis is referred to as the easily hydrolyzed organic acid-soluble phosphate. The difference between the concentrations of the organic acid-soluble phosphate and the easily hydrolyzed organic acid-soluble phosphate is referred to as the difficultly hydrolyzed organic acid-soluble phosphate.

Such analyses were carried out, in groups of ten animals, on normal, hyperthyroid, and hypothyroid rats after the administration of glucose, and on normal rats after the administration of sodium chloride. Animals were rendered hyperthyroid by the daily intraperitoneal injection of 0.1 mg. of thyroxine per 100 gm. of weight for 12 days prior to the absorption experiments. A second group of animals was rendered hypothyroid through

<sup>2</sup> Many of the intestines, particularly those of the thyroidectomized animals, contained a substance (possibly bile salts) which interfered with the estimation of the directly determined phosphate. The substance can be removed adequately by washing with two 5 cc. portions of a cold isotonic solution of sodium chloride. Such washings do not cause a perceptible change in the concentration of the acid-soluble phosphate.

surgical removal of the thyroid gland.<sup>3</sup> The operation was performed at least 3 weeks prior to the experiment.

In an effort to accentuate the differences obtained by the analyses of the mucosa of the whole intestine, a comparison was made of the concentrations of the several fractions of the acid-soluble phosphate in the mucosa of the upper quarter and the lower three-quarters of the intestine during the absorption of glucose at an accelerated rate (thyroxinized animal). In order to obtain sufficient mucosa from the upper portion of the intestine it was necessary to pool the samples of four animals. The data for the lower three-quarters likewise represent the results of the analyses of the pooled samples of four animals. Results are also reported for a similar experiment conducted on the normal animal during the absorption of sodium chloride.

### *Results*

The data obtained by analysis of the mucosa of the whole intestine in the four groups of animals are presented in Table I. In the next to the last column the acid-soluble organic phosphate is expressed as per cent of the total acid-soluble phosphate and in the last column the easily hydrolyzed organic acid-soluble phosphate is expressed as per cent of the organic acid-soluble phosphate. Inasmuch as no changes occurred in the concentrations of the 7 and 30 minute acid-soluble phosphates that were not accounted for by the changes in the concentration of the easily hydrolyzed (180 minute) acid-soluble phosphate, only the latter values will be considered in the discussion.

A comparison (Table I) of the concentrations of the several fractions of the acid-soluble phosphate of the intestinal mucosa during the absorption of glucose in the normal, thyroxinized, and thyroidectomized female rat shows the following. (a) Regardless of the rate of absorption of glucose, the organic acid-soluble phosphate constitutes an invariable portion (65 per cent) of the total acid-soluble phosphate. It follows that the small variations in the concentrations of the directly determined acid-soluble phosphate and the organic acid-soluble phosphate are accompanied by a comparable variation in the concentration of the total acid-

<sup>3</sup> The authors wish to acknowledge the debt to Dr. R. I. Pencharz for performing the thyroidectomies

soluble phosphate. The concentration of the organic acid-soluble phosphate in the case of the rapid absorption of glucose represents only a 12 per cent increase over the concentration of this fraction during the slow absorption of glucose. (b) Under the conditions of normal and retarded rates of absorption of glucose, the easily hydrolyzed organic acid-soluble phosphate forms a constant portion (26 per cent) of the organic acid-soluble phosphate. Accordingly, this fraction also constitutes a constant portion (17 per cent) of the total acid-soluble phosphate.

Under the condition of the rapid absorption of glucose, the easily hydrolyzed organic acid-soluble phosphate constitutes a slightly greater portion of both the organic acid-soluble phosphate and the total acid-soluble phosphate. The easily hydrolyzed fraction forms 29 per cent of the organic acid-soluble phosphate and 19 per cent of the total acid-soluble phosphate. Despite the fact that the concentrations of the organic acid-soluble phosphate and the easily hydrolyzed organic acid-soluble phosphate vary directly with the velocity of absorption of glucose, the differences are small and are probably non-significant.

A comparison of the concentrations of the several fractions of acid-soluble phosphate in the intestinal mucosa of the female rat during the absorption of sodium chloride and during the absorption of glucose shows the following. (a) In all cases of the absorption of glucose (accelerated, normal, and retarded rates), the organic acid-soluble phosphate constitutes a greater part (65 per cent) of the total acid-soluble phosphate than in the case of the absorption of sodium chloride (58 per cent). However, the difference in the concentrations of organic acid-soluble phosphate is small. The higher percentage of organic acid-soluble phosphate that accompanied the absorption of glucose was obtained in one case (thyroxinized animal) apparently through the conversion of a portion of the directly determined acid-soluble phosphate into the organic fraction, and in the other two cases (normal and thyroidectomized animals) through a diminution in the concentration of the directly determined fraction. (b) In contrast to the conditions that obtain as a result of the absorption of glucose, during the absorption of sodium chloride the concentration of the easily hydrolyzed organic acid-soluble phosphate amounts to only 55 mg. per cent and constitutes but 21 per cent of the or-

TABLE I

*Concentration and Percentage Composition of Several Fractions of Acid-Soluble Phosphate in Intestinal Mucosa of Rat under Different Experimental Conditions*

Ten animals were used for each experiment. The mean values and standard deviations for the concentration are given in mg. per cent (dry weight).

Experimental animals	Substance administered	Directly determined	7 min.	30 min.	180 min.	Difficultly hydrolyzed	Organic	Total	$\frac{\text{Organic}}{\text{Total}} \times 100$		$\frac{\text{Easily hydrolyzed}}{\text{Organic}} \times 100$	
									per cent	per cent	per cent	per cent
Thyroxinized	Glucose	153 $\pm$ 10	29 $\pm$ 7	59 $\pm$ 14	84 $\pm$ 13	202 $\pm$ 9	286 $\pm$ 17	439 $\pm$ 14	65.0	29.4		
Normal	"	139 $\pm$ 18	26 $\pm$ 10	49 $\pm$ 9	69 $\pm$ 11	194 $\pm$ 21	264 $\pm$ 29	402 $\pm$ 45	65.7	26.2		
Thyroidectomized	"	140 $\pm$ 16	28 $\pm$ 8	48 $\pm$ 10	65 $\pm$ 13	187 $\pm$ 25	252 $\pm$ 20	392 $\pm$ 39	64.3	25.8		
Normal	Sodium chloride	185 $\pm$ 13	28 $\pm$ 16	37 $\pm$ 16	55 $\pm$ 16	203 $\pm$ 22	257 $\pm$ 27	442 $\pm$ 21	58.2	21.4		

TABLE II

*Concentration (in Mg. Per Cent, Dry Weight) and Percentage Composition of Several Fractions of Acid-Soluble Phosphate in Upper and Lower Portions of Mucosa of Intestine of Rat under Different Experimental Conditions*

Experimental animals	Substance administered	Portion of intestinal mucosa (pooled samples of 4 animals)	Directly determined	180 min.	Difficultly hydrolyzed	Organic	Total	$\frac{\text{Organic}}{\text{Total}} \times 100$		$\frac{\text{Easily hydrolyzed}}{\text{Organic}} \times 100$	
								per cent	per cent	per cent	per cent
Thyroxinized	Glucose	Upper $\frac{1}{2}$	122	89	206	295	417	71	30.2		
		Lower $\frac{1}{2}$	139	80	212	292	431	67.8	27.4		
	Sodium chloride	Upper $\frac{1}{2}$	159	58	161	219	378	58	26.4		
		Lower $\frac{1}{2}$	165	60	173	233	398	60	25.8		

ganic acid-soluble phosphate and 12.5 per cent of the total acid-soluble phosphate.

According to Verzár and Wirz (5) the absorption of glucose is greater in the upper portion than in the lower portion of the small intestine. Perhaps the small change in the concentration of the easily hydrolyzed organic acid-soluble phosphate that results from changes in the rate of absorption of glucose represents a significant variation in the concentration in a small portion of the intestine that is masked by the acid-soluble phosphate that is present in the remaining portion. If this is so, then there should be a significant difference in the concentration of this fraction in the mucosa of the upper one-quarter and the lower three-quarters of the intestine during the absorption of glucose at an accelerated rate (thyroxinized animal).

The data (Table II) show that during the rapid absorption of glucose there is no significant difference in the concentrations of the several fractions of the acid-soluble phosphate in the mucosa of the upper quarter and lower three-quarters of the intestine. The data for the analyses of the mucosa of the upper and lower portions of the intestine during the absorption of glucose and sodium chloride confirm the results that were obtained for the mucosa of the whole intestine.

#### DISCUSSION

Studies conducted on the intestinal absorption of the monosaccharides have shown that glucose and galactose, sugars which are susceptible to phosphorylation, are absorbed at a rate that is characteristic for each sugar and is, within rather wide limits, independent of the concentration of these sugars in the lumen. Mannose, sorbose, and xylose are absorbed at a rate that is proportional to the concentration of the sugars. The selectively absorbed hexoses are absorbed at a greater rate than other sugars (6).

According to the phosphorylation theory of intestinal absorption, the selective absorption of glucose and galactose is dependent upon the conversion of these hexoses, presumably within the epithelial cells of the intestinal mucosa, to hexosephosphates. In this manner there is obtained a steep gradient for the diffusion of the hexoses across the epithelial cell membrane that is proximal



to the lumen of the intestine. It seems expedient to consider that the absorption of a substance across the intestinal barrier involves a passage through the epithelial cell membrane that is proximal to the lumen, into the protoplasm of the cell, and through the cell membrane that is distal to the lumen. Inasmuch as the free hexose is recovered in the blood, hydrolysis of the hexose-phosphate must occur either within or without the cell. It is attractive to consider that hydrolysis occurs outside of the epithelial cell membrane that is distal to the lumen of the intestine; possibly, in the area between the membrane and the capillary wall. In this way there is obtained a steep gradient for the diffusion of the ester out of the cell. According to this theory, the concentration of any ester that may occur as a result of phosphorylation in the mucosa would be governed to a large extent by the following factors: (a) the rate of formation of the ester, (b) the concentration of the ester that is necessary to establish a gradient for the diffusion of the ester through the cell protoplasm, and (c) the rate of disappearance of the ester. In the case wherein the velocity of formation and the velocity of disappearance are equal, the concentration of the hexosephosphate will be governed by the conditions of the steady state. If the velocities are not equal, conditions *may* exist wherein changes in the concentration of the esters may accompany changes in the velocity of the absorption of glucose.

The results of the work herein reported show that variations in the velocity of the absorption of glucose, as effected by the thyroid gland, do not cause any significant changes in the concentrations of several fractions of the acid-soluble phosphate of the mucosa of the whole intestine of the rat. A comparison of the concentration of the several fractions of the acid-soluble phosphate in the mucosa of the upper portion of the intestine and the lower portion of the intestine during the rapid absorption of glucose confirms the above findings. There is no significant difference in the concentration of phosphates in the different portions of the mucosa during the rapid absorption of glucose.

It may be concluded that although the absorption of glucose is accompanied by a slight increase in the percentage of the acid-soluble phosphate esters over that observed during the absorption of sodium chloride, changes in the rate of absorption of glucose,

as influenced by the thyroid gland, have little or no effect on the concentration of these esters.

The lack of correspondence between the concentration of the acid-soluble phosphate esters and the velocity of absorption of glucose neither supports nor denies the validity of the hypothesis that phosphorylation is a process that is concerned with the selective absorption of glucose and galactose. For it must be assumed that the concentration of such esters depends upon the rate of formation and the rate of disappearance. Accordingly, a difference in the rate of turnover of the acid-soluble phosphates need not be accompanied by a change in concentration.

#### SUMMARY

A study of the concentrations of several fractions of the acid-soluble phosphate in the intestinal mucosa during the absorption of glucose at various rates neither supports nor invalidates the hypothesis that phosphorylation is a process concerned with the selective absorption of certain hexoses.

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# THE RELATION OF THE PHOSPHORUS TURNOVER OF THE BLOOD TO THE MINERAL METABOLISM OF THE CALCIFIED TISSUES AS SHOWN BY RADIOACTIVE PHOSPHORUS\*

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Detailed data on the phosphate turnover of rat blood, bones, and teeth have been reported (1). The interpretations were based on phosphate changes calculated as percentages of the original dose of marked phosphorus found in each gm. of tissue at a specified time after administration. Such calculations give an adequate description of the findings but do not facilitate an understanding of the metabolic relations between the tissues. For example, although it has long been considered a valid concept that the blood minerals are in some sort of equilibrium with the corresponding bone minerals, by the above manner of calculation it would appear that in 48 hours blood containing 0.20 per cent of the total dose per gm. deposits 10 per cent of the total dose in bone. If such an amount of marked phosphorus entered the bone as new calcification, one-fifth of the total bone would have to be deposited in 24 hours, which is absurd. However, the relation between blood and bone phosphates may be explained by calculating the changes in terms of the  $P^*:P$  ratio,<sup>1</sup> i.e., the fraction of the dose of marked phosphorus found per mg. of total phosphorus in the various tissues.

The first important point to come from this calculation is that

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<sup>1</sup> The asterisk indicates radioactive phosphorus. The number of  $P^{32}$  atoms is small enough to be negligible compared to the number of  $P^{31}$  atoms; therefore the ratio  $P^*$  to total P is equivalent to the ratio  $P^*$  to inert P, or  $P^{32}:P^{31}$ .

the marked phosphorus concentration initially is much higher in the blood than in the calcified tissues. Thus, 24 hours after the ingestion of the dose, 1 gm. of blood contains 0.21 per cent of the  $P^*$  dose and 0.465 mg. of total P, equivalent to a  $P^*:P$  ratio of 0.46; while 1 gm. of epiphyseal bone (inorganic part) contains 10.3 per cent of the  $P^*$  dose and 190.0 mg. of total P, equivalent to a  $P^*:P$  ratio of 0.07. Thus, the blood ratio is approximately 6.5 times that of the epiphyseal bone.

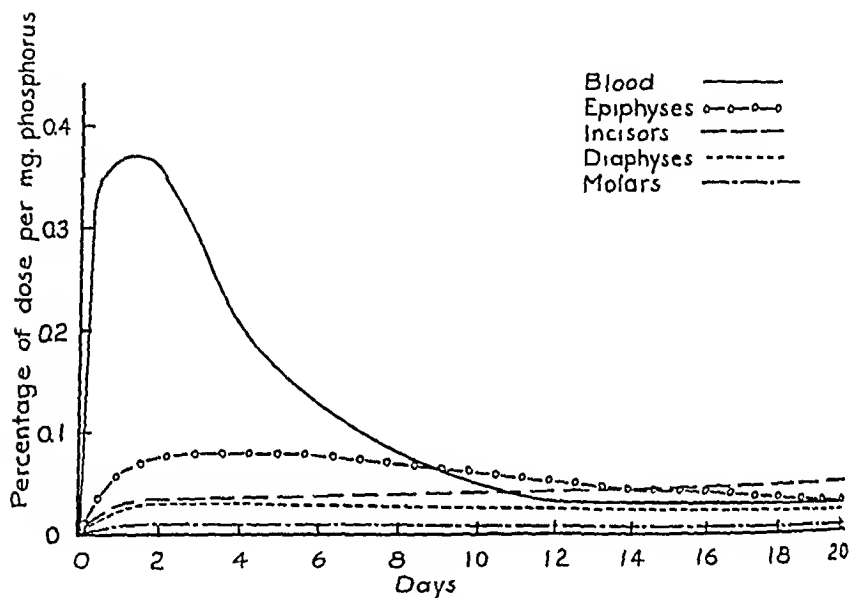


FIG. 1. Concentration of marked phosphorus per mg. of total phosphorus in the tissue. Note that the concentration in none of the calcified tissues reaches that of the blood before 8 days.

From the percentage dose *versus* time curves given by Manly and Bale in their Fig. 1 (1), values for the  $P^*:P$  ratio were calculated for blood, epiphyses, diaphyses, molars, and incisors. A study of these  $P^*:P$  ratio *versus* time curves (Fig. 1) reveals three significant facts. (1) During the first 4 days the blood ratios are high and the bone ratios low. Although the ratios for the calcified tissues rise rapidly during the first 24 hours, the values remain below the blood value for the ensuing 6 days. It is reasonable to inquire why the rise in ratio of the calcified tissues is checked so sharply if equilibrium with the blood is taking place. (2) Not only do the bone ratios fail to reach the blood ratios but

in the case of the epiphyses and diaphyses, marked phosphorus is lost in the face of a high  $P^*:P$  ratio in the blood. (3) The concentrations in the blood and epiphyses are not equal until the 12th day and it apparently requires even longer for equilization to take place between blood and diaphyses.

To set up a mechanism by which these complicated changes can be described requires certain assumptions. First, the marked phosphorus is assumed to be in rapid and continuous equilibrium with the various phosphate fractions of the blood, so that all the fractions of blood phosphorus contain identical ratios of  $P^*:P$ .<sup>2</sup> Consequently the  $P^*:P$  ratio of the *whole* blood can be taken as a measure of the  $P^*:P$  ratio of that fraction of the blood phosphate which is in equilibrium with bone.

The second assumption is that calcified tissues are composed physiologically of two portions, one of which rapidly comes to equilibrium with certain blood minerals. This portion will be designated as labile bone. The other portion reaches equilibrium slowly and, by comparison, exhibits negligible exchange with these blood minerals. It will be designated as stable bone. This concept does not denote any anatomical location of the two portions.

When these two assumptions are combined, the equilibrium between blood and labile bone will be characterized by identical  $P^*:P$  ratios in the two tissues. On the other hand the exchange between blood and stable bone is sufficiently slow so that the  $P^*:P$  ratio of stable bone will lag far behind any changes in the blood ratio.

With these criteria, the three points described above will be treated in order.

*Apparent Discrepancy between Initial High Blood and Low Bone  $P^*:P$  Ratios*—The limited rise for bone<sup>3</sup> values results from averaging the  $P^*$  content of the labile fraction with that of the proportionately larger stable fraction. At the end of 24 hours, the  $P^*:P$  ratio for blood is 0.36, for epiphyseal bone 0.06; there-

<sup>2</sup> Some experimental evidence is available to support this assumption; e.g., the equality of  $P^*$  distribution between total blood P and blood phospholipid P (2).

<sup>3</sup> Wherever "bone" is referred to in the following discussions, it includes only the epiphyseal or diaphyseal bone on which data are available.

fore, about one-sixth of the bone may be considered as labile with a ratio equal to that of blood, and the remaining five-sixths as stable with as yet negligible quantities of marked phosphorus (see Table I). Thus the sudden rise in marked phosphorus in the epiphyseal bone is to be ascribed not to a deposition of phosphorus but to an exchange in which only one-sixth of the phosphorus atoms of the bone have come to equilibrium with the blood. On this basis the discrepancy between the high blood and low bone ratios is only apparent and not real.

This explanation, however, raises a question as to why the ratios for the calcified tissues do not parallel the blood ratios throughout the whole experiment. Assuming a labile portion of bone in equilibrium with the blood, a sharp fall in the bone ratio would be expected from the 2nd to the 8th day. Actually, as the blood levels fall, the values for the total bone remain nearly constant. This may be attributed to an increase in the  $P^*:P$  ratio in *stable* bone resulting principally from new calcification. The necessary amount of daily phosphorus deposition to give a curve agreeing closely with that for diaphyses in Fig. 1 is approximately  $1/50$  of the total amount of phosphorus already present in the bone. This corresponds to a daily deposition of about 10 mg. of phosphorus, which is almost exactly the value obtained by calculation from the data given by Donaldson (3) on skeletal calcification rates in young male rats.

*Loss of Marked Phosphorus from Bone in Contact with Blood of Higher  $P^*:P$  Ratio*—From the 2nd day the  $P^*:P$  ratio in the blood falls, first rapidly and then slowly, causing an equivalent decrease in the ratio in the labile bone. Since, in the period from the 2nd to the 4th day, the labile bone contains a major portion of the marked phosphorus in the bone, the ratio for whole bone also falls. However, the curve for bone ratios does not continue to decrease because increasing quantities of radioactive phosphate are present in the stable portion. Thus, about the 5th day the labile and stable bone contribute equally to the total marked phosphorus content. However, even at this time the  $P^*:P$  ratio in the stable bone is much lower than that in the labile bone or in the blood. Therefore, the decrease in the ratio in the labile portion of the bone results in a decrease in the  $P^*:P$  ratio for whole bone.

*Significance of Convergence of Curves for Blood and Bone*—The P\*:P ratio in the blood falls very slowly from the 12th to the 20th day, with a concomitant decrease in the ratio of the labile bone. However, the ratio in stable bone is increasing and the P\*:P ratio of each daily increment is the same as the current blood ratio. Hence, the curves for blood and bone must converge and finally cross; this convergence is seen in the curves for epiphyses, incisors, and diaphyses, and a crossing in the curves for incisors and perhaps epiphyses.

The relative amounts of labile to stable calcification differ widely in epiphyses, diaphyses, incisors, and molars, as is shown in Table I; the percentage figures were obtained by setting the ratio in the labile bone equal to that in the blood. Stable bone

TABLE I  
*Magnitude of Labile Fraction of Various Tissues*

	Fraction of whole tissue which is labile	Per cent of total marked phosphorus present in labile portions	
		1 day	20 days
Epiphyses.....	1/6	95	21
Diaphyses.....	1/12	90	11
Molars.....	1/50	87	8
Whole incisors.....	1/12	73	3

The percentage figures for total P\* in the labile portions are taken from the summed curves (see the text).

was assumed to add equal daily increments<sup>4</sup> of phosphorus possessing the P\*:P ratio current in the blood. In this fashion two curves were derived, which were adjusted so that the sum would fit the experimental points for the 1st and 20th days. From the adjusted curves the relative contributions of the labile and of the stable portions can be compared with the total marked phosphorus content. On the 1st day 95 per cent of the total marked phosphorus in the epiphyses is in the labile portion. The other tissues range down from this value to 73 per cent in the labile portion of the whole incisors. On the 20th day, 21 per cent of the total marked phosphorus in the epiphyses is in the labile portion, while only 3 per cent is in the same portion of the whole incisors.

<sup>4</sup> "Accretion rate."



In comparison with the other calcified tissues the labile portion of the epiphyses is the largest (one-sixth) and has the highest percentages of the total marked phosphorus; in addition, its accretion rate is higher than for the diaphyses. In other words, the epiphyses are the site of the most active mineral metabolism. The relative magnitudes of the labile portions of the diaphyses and of the whole incisors are comparable even though the accretion rate of the incisors is nearly 3 times that of the diaphyses. This is to be expected from the known high rate of calcification of the incisors, which is also shown by the very low contribution of the labile portion at 20 days. The labile portion of the molars is minute; this together with a low accretion rate brings the  $P^*:P$  ratio for the molars to only one-fifth of that of the blood even at 20 days. Although the relative importance of the labile portion of the molars is numerically quite similar to that of the diaphyses, the mineral metabolism of the molars is small compared to that of the diaphyses. This indicates that similar processes differing in degree are occurring, in agreement with the concept that dentin is analogous to dense cortical bone.

#### SUMMARY

A rational explanation of the behavior of the calcified tissues following the administration of a dose of radioactive phosphorus has been evolved on the basis of two assumptions; first, that all forms of phosphorus in the blood are in equilibrium, and second, that bone is composed of a labile portion in equilibrium with the blood, and a stable portion receiving phosphorus in an incremental fashion. From a knowledge of the  $P^*:P$  ratio in blood and in bone, one may calculate the relative amounts of labile and stable bone. With these values three theoretical considerations are derived from the experimental results.

1. Immediately following the administration of radioactive phosphorus, the high blood  $P^*:P$  ratio is accompanied by an identical ratio in the labile bone. The labile bone, however, constitutes a sufficiently small portion of the whole bone to make the total bone  $P^*:P$  ratio much lower than that of blood.

2. The blood  $P^*:P$  ratio falls rapidly during the 2nd to 4th day, with an equal fall in the ratio in the labile bone. In this period, the stable bone has added a small daily increment of new

calcification which has the  $P^*:P$  ratio currently in the blood; this amount is insufficient to prevent a fall in the total bone  $P^*:P$  ratio.

3. By about the 5th day, the stable bone, owing to continuous accretion of marked phosphorus, has a  $P^*$  content equal to that of labile bone. After the 5th day, although the ratio falls slowly in the labile bone, the ratio in the stable bone and consequently in the whole bone increases. By the 15th day, in rapidly calcifying tissues, this increase gives a demonstrably higher  $P^*:P$  ratio in the whole tissue than in the blood.

From these calculations, a quantification is made of certain phases of mineral metabolism of the calcified tissues. The numbers expressing physiological processes previously known qualitatively help to explain the chemical changes which occur in calcifying tissues. These numbers are used to determine the magnitude and kind of certain physiological elements of calcification in a given tissue and to compare these functions in various tissues.

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# INTERPRETATION OF THE SACCHAROGENIC ACTION OF DIASTASE ON THE BASIS OF SUBSTRATE COMPETITION\*

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Since the reducing substances formed in diastatic reactions normally represent mixtures of glucose, maltose, and non-fermentable reducing matter (1), we estimated in this study the quantities of each of the three constituent groups separately by means of methods previously described (2). In one portion of the reaction mixture the total reducing power was determined (*a*); another portion was fermented with washed bakers' yeast at alkaline reaction (pH 8.0 to 8.4) for 30 minutes before determination of the reducing power (*b*); a third portion was fermented at unmodified reaction for 2 hours and the residual reduction (*c*) was then determined.  $a - b$  = copper reduced by glucose;  $b - c$  = copper reduced by maltose;  $c$  = copper reduced by non-fermentable substances.

The amount of maltose was calculated from the reduced copper by multiplying the corresponding glucose equivalent by the factor 1.55, as we have found that, with the "high alkalinity" copper reagent employed in this work, 1.55 mg. of maltose reduce the same amount of copper as 1 mg. of glucose. The quantity of the non-fermentable reducing substances, referred to hereafter as reducing dextrans, was expressed in terms of maltose.

The objective of this work was exploration of the character of possible new information that can be obtained by our method of tripartite analysis, rather than a comprehensive study of the kinetics of diastase action. Accordingly we report in this paper only on the influence of changes in a limited number of factors, such as time, enzyme concentration, substrate concentration, and dilution. The quality of substrate, temperature, electrolyte

\* This work was aided by a contribution from Mr. Hugo F. Urbauer.

concentration, and hydrogen ion concentration were, on the basis of close scrutiny, standardized and kept unaltered throughout these experiments.

*Substrate*—Soluble starches, as shown by Sherman and Baker (3) and by several other workers, yield variable results. Lüers and Wasmund (4) called attention to the profound influence that heating of starch paste under pressure exerts upon the course of diastatic reactions. In order to avoid the influence of these variable factors, we employed as substrate starch paste, prepared of a good commercial grade of corn-starch, at 100°. But even slight natural variations occurring in unmodified starches suffice to affect the results of enzyme action. To obviate this source of variability, O'Sullivan (5) and Sherman, Walker, and Caldwell (6) subjected the starch to washing with dilute acid and alkali. We found that washing corn-starch with 0.01 N hydrochloric acid and then with water served the purpose satisfactorily.

*Temperature*—Our observations were uniformly made at 40°. This is not the temperature at which the maximum amount of reducing matter is produced. One should be aware of the fact, however, that in experiments extending over long reaction periods, the maximum amount of reducing matter is produced at lower temperatures than in brief reaction periods; *i.e.*, the longer the reaction time, the lower the optimal temperature. This is due to the fact that elevation of the temperature exerts two counter-current effects: while it enhances enzyme activity, it also accelerates the rate of inactivation of the enzyme. Since the temperature coefficient of the latter process is higher than that of the first, time brings the destructive effect to the fore. Thus, if one wanted to employ optimum temperature, this would have to be sought out for every particular reaction period. To avoid this complication, we conformed with general usage in adopting 40° as the standard temperature.

*Electrolyte Concentration*—Available data as to the concentration of NaCl at which the reaction proceeds at a maximum rate are contradictory. Discrepancies are particularly great in figures concerning diastases of different origin. We found it necessary, therefore, to determine the optimum NaCl concentration for blood and urine diastases, the principal objects of our studies. May it suffice to state without the presentation of de-

tailed data that for the diastatic activity of human blood plasma, urine, and saliva, 0.2 to 0.4 per cent represents the optimum range of NaCl concentrations.

*Hydrogen Ion Concentration*—Blood, urine, and salivary diastase exert maximum saccharogenic activity at pH 6.8 to 7.4, if all other conditions affecting the reaction are constant. In view of the width of this range we deemed it sufficient to adjust the pH of the enzyme solutions to 7.0 to 7.2 by the addition of acid or alkali, without incorporating any extraneous buffer solution in the reaction mixtures.

### *Effect of Time and Enzyme Concentration*

In the construction of time curves of the saccharogenic process, mixtures of starch paste and enzyme solution were set up in batches sufficiently large to allow the frequent withdrawal of samples for analysis during periods of several hours. The diastase employed in this group of experiments was a preparation isolated from human urine by precipitation with 3 volumes of alcohol. After centrifugation and decantation, the precipitate was dissolved in water, subjected to dialysis against running water for several hours, and centrifuged to remove insoluble particles. Precipitation with alcohol and removal of water-insoluble constituents were repeated. In this manner the diastase content of large quantities of urine was concentrated in a small volume of water, to which 0.6 per cent NaCl was added. Such preparations were kept in a refrigerator for several days without appreciable deterioration in their activity.

Four dilutions of a stock solution were prepared, with 0.6 per cent NaCl as diluent. Denoting the enzyme concentration of the weakest solution as  $C$ , we have termed the concentrations of the other three solutions  $5C$ ,  $10C$ , and  $50C$  respectively. For observations of the effect of time upon the course of the reaction, the enzyme solution was prewarmed to  $40^{\circ}$  and mixed with an equal volume of 3 per cent starch paste, also warmed in advance to  $40^{\circ}$ . The mixture was incubated in a constant temperature water bath. Samples for analysis were withdrawn at various intervals of time up to 3 hours. In order to stop enzyme action, the samples were quickly heated to about  $80^{\circ}$ , then cooled, and kept overnight in the refrigerator. The unliquefied residue of the

starch, which was considerable in the instance of the two weaker enzyme solutions, formed in the cold a sediment which was eliminated by centrifugation. In the supernatant fluid glucose, maltose, and reducing dextrans were determined.

*Experiment 1*—This experiment was executed with enzyme concentration *C*. The results, presented in Fig. 1, show that the hydrolytic reaction products during the first 15 minutes consisted entirely of non-fermentable polysaccharides. Maltose appeared at the end of 60 minutes, and then only in a small quantity that would have eluded detection without the aid of a sensitive micro-method; after 3 hours maltose still represented no more than 21 per cent of the aggregate reducing power. Of glucose no trace appeared throughout the 3 hours.

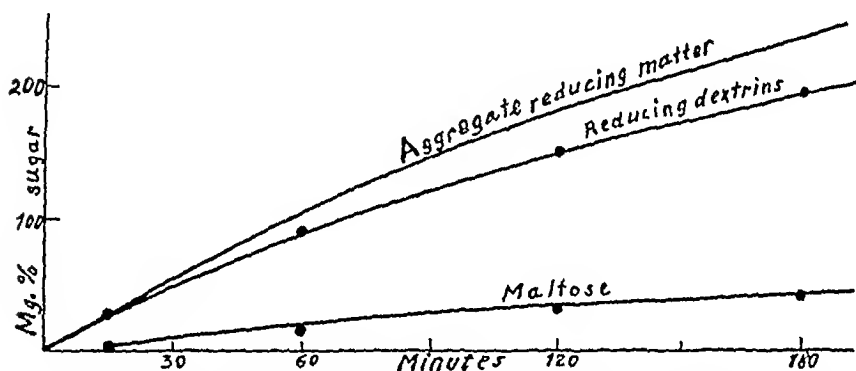


FIG. 1. Time curves of the saccharogenic reaction of diastase of concentration *C* (concentration of weakest solution).

*Experiment 2*—In this experiment, in which the enzyme concentration was 5 *C*, a different picture was unfolded. As shown by the curves in Fig. 2, the reducing dextrans still predominated, but maltose was already detectable after 15 minutes, and after 3 hours was accountable for 38 per cent of the reducing power of the reaction mixture.

In contrast to Experiment 1, glucose made its appearance in the 3rd hour of the reaction. True, its quantity was so small that it could not be estimated without the use of the selective fermentation technique and of an adequately controlled micromethod of sugar analysis; yet it was by no means negligible.

*Experiment 3*—Further augmentation of the enzyme concentra-

tion effected a further shift in the mutual relationship of the reaction products. As may be seen in Fig. 3, besides reducing dextrins and maltose, glucose appeared quite early; namely, after the first half hour of the reaction. A new feature in these curves is the marked drop in the amount of reducing dextrins during the second half of the 3 hour period, after having reached a maximum between the 1st and the 2nd hour. At the end of 3 hours maltose became the predominant reaction product, accounting for 51 per

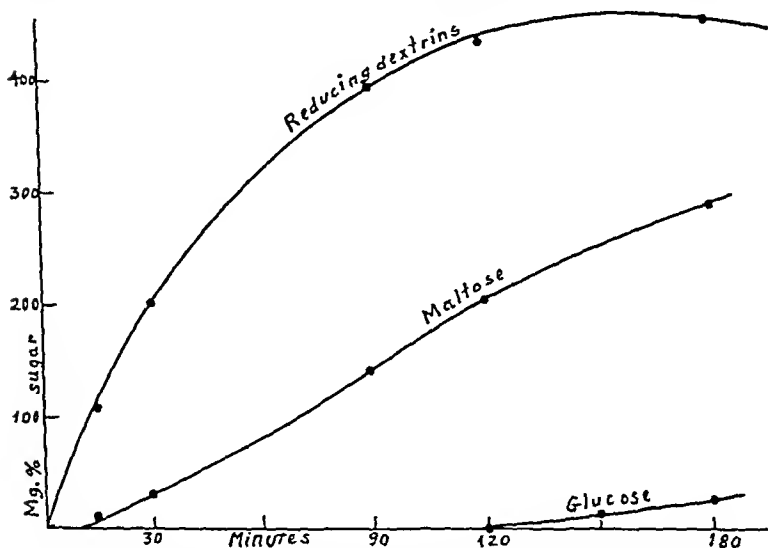


FIG. 2. Time curves of the saccharogenic reaction of diastase of concentration 5 C.

cent of the aggregate reducing power. Glucose at this point accounted for 6.4 per cent of the reduced copper.

*Experiment 4*—In the fourth experiment of the series, carried out with the highest enzyme concentration, the shift in the quantitative relationship of the several reducing substances was greatly accentuated. Fig. 4 shows that maltose was produced from the beginning at only a slightly lower rate than the reducing dextrins; after 15 minutes, however, the rate of maltose formation outstripped that of the production of reducing dextrins, so that the quantity of the latter began to drop and continued to decline



to the end of the 3 hour period. Glucose was detectable as early as the 15th minute of the reaction and attained well measurable quantities in 30 minutes. At the end of the 3 hour period maltose accounted for 71 per cent, glucose for about 10 per cent of the copper-reducing power of the reaction mixture. The shift is accentuated by the fact that after as brief a reaction period as 30 minutes, 64 per cent of the reducing power was due to maltose and 5 per cent to glucose.

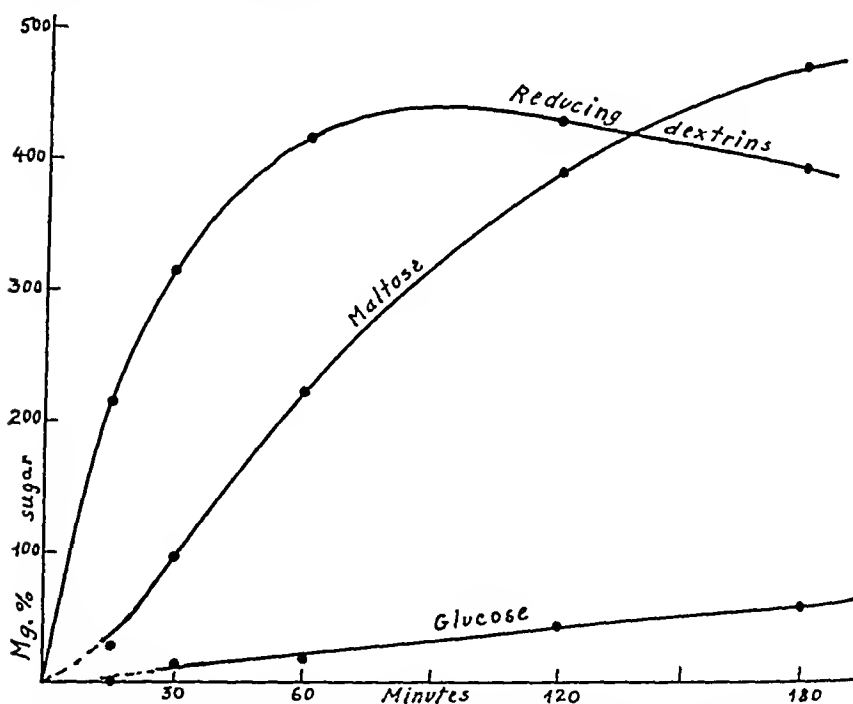


FIG. 3. Time curves of the saccharogenic reaction of diastase of concentration 10 C.

This is perhaps an opportune place for pointing out a possible source of wide-spread contradictions and confusing assertions concerning the identity of the reaction products obtained with diastases of various origin. Any investigator, working under the conditions of our Experiment 1 (Fig. 1), may conclude that the diastase involved hydrolyzes starch to reducing dextrins and maltose but produces no glucose at all. This conclusion could be drawn, however, only if the experiment were extended to at least 3 hours; but were it terminated after a period of 30 minutes, the

observer could only state that the enzyme produces non fermentable reducing matter exclusively. Should that observer hold the view that dextrins are hexosans that possess no reducing power, he would conclude that the non-fermentable reducing matter is "isomaltose" and represents the sole end-product of the reaction. The literature is certainly replete with assertions of this kind.

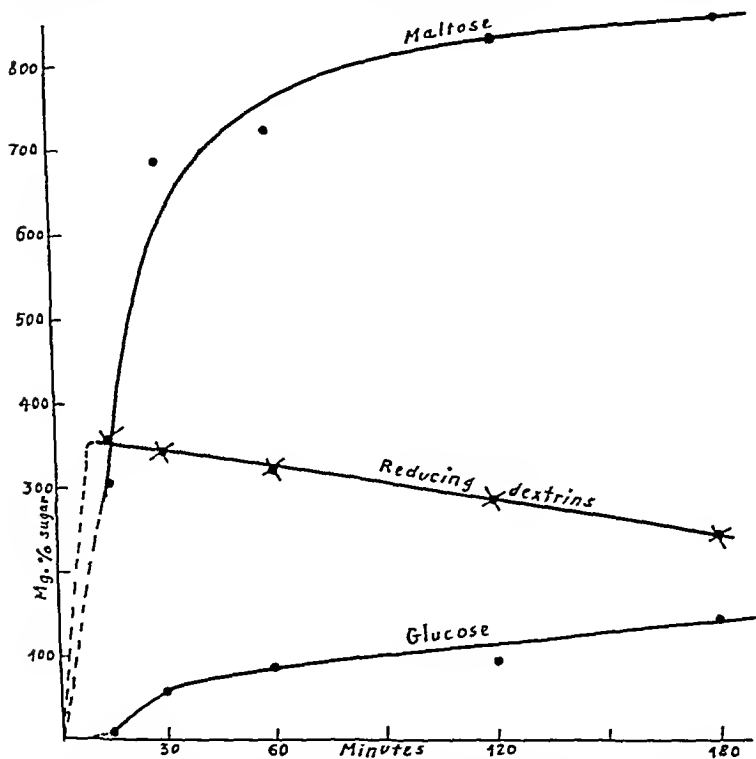


FIG. 4. Time curves of the saccharogenic reaction of diastase of concentration 50 C.

Other workers, incidentally, dealing with conditions like those in our Experiment 2 (Fig. 2), would be led to the conclusion that maltose is the end-product of the diastatic reaction and that glucose is not even detectable among the reaction products.

On the basis of our four experiments, however, when they are considered collectively one arrives at entirely different conclusions.

Figs. 1 to 4 make it evident that one and the same enzyme is capable of producing either non-fermentable reducing substances (dextrins) only, or reducing dextrins plus maltose, or in addition to these glucose. The result is dependent upon the conditions that obtain in the reaction, such as enzyme concentration, reaction time, and others that need not be discussed on this occasion.

Glucose is undoubtedly a normal product of diastatic reactions. The contention, commonly voiced as a self-evident fact, that glucose is formed by maltase, which is contained in diastase preparations as a contaminant, has no foundation whatsoever in experimental evidence. On the contrary, Sherman's diastase preparations, which surpassed in purity any other product thus far described, did produce glucose from starch (7). It must be pointed out, moreover, that our diastase preparations, which do produce glucose from starch, invariably fail to act on maltose as a substrate, indicating that they do not contain maltase.

It has been our consistent observation that glucose is invariably formed in diastatic reaction mixtures, but that it emerges only after the bulk of the unaltered starch and erythro-dextrins has disappeared. The mother substance of glucose then is to be sought among the achroo-dextrins of rather small molecular size.

The question arises here whether or not various hexosans (non-reducing dextrins), of the nature of Schardinger's crystalline dextrins, occur among the normal diastatic split-products of starch. Our observations have not furnished definite information on this point. As previously reported (1), fractionation of the reaction products yields an array of non-fermentable polysaccharides of variable copper-reducing power. None of these represents a chemical entity, since each can be further partitioned into several fractions, some of which possess higher, others lower reducing power than the mother substance. With relatively low concentrations of alcohol and numerous reprecipitations, it is possible to isolate dextrins that show very low reducing power, so low in fact that when analyzed with moderately sensitive methods, the reducing power may fall within the limits of technical errors and in consequence appear negligible. Using sensitive micromethods, however, we are forced to conclude that none of the dextrins we have analyzed can be regarded as definitely devoid of copper-reducing power.

*Effect of Substrate Concentration*

The fact that glucose is not produced by diastase so long as substantial amounts of starch and erythrodextrins are present in the reaction mixture seems to be adequately explained by the observation that diastase preferentially combines with polysaccharides of colloidal structure (8) and thus becomes available for reactions with dextrins of smaller molecular size only after far reaching degradation of the colloids has been effected. Satisfactory support for this concept was obtained in experiments in which the enzyme concentration was kept constant while the concentration of the starch was varied.

TABLE I

*Effect of Substrate Concentration*

Time, 30 minutes; temperature, 40°.

Starch concentration	Reducing dextrins calculated as maltose	Maltose	Total reducing matter	
			Amount	As percentage of starch
<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
0.10	32.5	12.5	45.0	45.0
0.25	42.0	12.0	54.0	21.6
0.50	51.2	9.3	60.5	12.1
0.75	56.6	8.1	64.1	8.5
1.00	57.4	7.7	65.1	6.5
2.00	57.8	7.2	65.0	3.2
3.00	49.6	3.1	52.7	1.8

In Table I is presented such an experiment. The diastase solution in this instance was blood plasma. Identical quantities of plasma were incubated with identical volumes of starch pastes, the starch content of which ranged from 0.1 to 3.0 per cent. As may be noted in the fourth column of Table I, the quantity of the reducing matter increases with increasing starch concentrations until the latter passes the 0.5 per cent mark; then a plateau of maximum reduction values persists, followed by a drop after the starch concentration has exceeded 2.0 per cent. We wish to direct attention to the fact that when augmentation of the substrate concentration increases the rate of production of reducing matter, maltose has no share in this increase. On the contrary,

the amount of maltose drops by 42 per cent (dropping to 7.2 mg. from 12.5 mg.), while at the same time the aggregate reducing power of the reaction mixture rises from 45.0 mg. to 65.0 mg., an increase of 44 per cent. The increment is due to reducing dextrins, which show a rise from 32.5 to 57.8 mg., or 78 per cent.

This phenomenon indicates a competition of several substrates for the enzyme, a competition in which diastase preferentially reacts with large colloidal aggregates. It selectively combines with unaltered starch as long as the latter presents itself in abundance, and hydrolyzes the starch to reducing dextrins. The greater the excess of starch in the reaction mixture, the less of the enzyme is available for combination and reaction with the dextrins, and in consequence the smaller is the quantity of maltose produced. The contrast in our experiment is most conspicuous when the products of the reactions with 0.1 and 3.0 per cent starch are compared: while the amount of reducing dextrins is increased from 32.5 mg. to 49.6 mg. as the concentration of starch mounts, maltose drops from 12.5 mg. to 3.1 mg., an almost negligible quantity. (Here again it may be pointed out that experiments in which weak enzyme preparations react with large amounts of starch may give rise to the erroneous conclusion that the end-product of the reaction is a non-fermentable reducing substance, an "isomaltose.")

In this experiment glucose was produced only with 0.1 per cent concentration of starch, but only in a small quantity which eluded accurate estimation. It probably was formed at the terminal phase of the reaction when the color reaction with iodine showed that no starch and but little of erythrodextrins were present, and diastase presumably became available for combination with small molecules of reducing dextrins.

### *Effect of Dilution*

If the assumption that diastase has a general tendency to combine preferentially with larger polysaccharide molecules or colloidal aggregates is correct, then the fall in the rate of reaction that is caused by simple dilution of reaction mixtures with water should be greater in the production of maltose than in the production of reducing dextrins, and again greater in the formation of glucose than in that of maltose. Experiments to probe this concept yielded confirmatory results.

In Table II is presented an experiment performed with the quantities of enzyme and starch constant, but with the reaction mixture dispersed in increasing volumes of fluid in order to enhance the dissociation of the enzyme-substrate complexes. If in the conventional manner only the aggregate reducing power of the reaction mixtures is scrutinized, the results confirm the known fact that extensive dilution diminishes the rate of the reaction. 4-Fold dilution had but a scarcely discernible effect in both the 30 and the 60 minute periods, but 16-fold dilution promoted the dissociation of the enzyme-substrate combination to such an extent that it effected a distinct decrease in the rate of the reaction.

TABLE II

*Effect of Dilution upon Quantitative Relationship of Reducing Substances Formed in Diastatic Reactions*

The reaction mixture contained identical amounts of enzyme and of substrate, only in different volumes of solution; the concentration of NaCl was 0.3 per cent throughout.

Reaction time	Volume of reaction mixture	Glucose	Maltose	Reducing dextrins	Total reducing matter	Changes in quantity caused by dilution		
						Glucose	Maltose	Reducing dextrins
min.	cc.	mg.	mg.	mg.	mg.	per cent	per cent	per cent
30	100	4.5	29.3	54.4	88.2	0	0	0
	400	4.0	26.1	56.2	86.3	-12.5	-11.0	+4.0
	1600	2.4	16.2	57.6	76.2	-47.0	-45.0	+5.9
60	100	8.0	58.2	50.0	116.2	0	0	0
	400	6.8	53.3	55.3	115.1	-17.5	-8.4	+10.6
	1600	3.3	37.8	59.6	100.7	-59.0	-35.0	+19.2

In the light of the results obtained by our tripartite analysis, however, an entirely different picture was revealed. As may be noted, the rate of formation of reducing dextrins not only failed to decline but showed a definite rise when the dilution of the reaction mixture was increased. This implies the fact that the decline in the aggregate reducing power was due to a drop in the combined reducing power of maltose and glucose, a drop greater than the rise in the reducing power of the dextrins. The figures given in Table II show that at the end of a reaction period of 60 minutes, 35 per cent less maltose and 59 per cent less glucose were

formed when the dilution was increased 16-fold, while the amount of reducing dextrins increased by 19.2 per cent. This is a clear instance of substrate competition in which polysaccharides of larger molecular size gain preference in combining with the enzyme, at the expense of the smaller split-products of starch. Increase in the dilution of the reaction mixture enhances the dissociation of the substrate-enzyme complex the more effectively, the smaller the molecule of the substrate. Thus, by increasing the extent of dilution, one increases that part of the enzyme which is available for reaction with the unhydrolyzed and slightly hydrolyzed colloidal portion of the substrate, and in consequence the rate of the reaction that produces reducing dextrins is increased at the expense of maltose and glucose formation.

It may be noted on further inspection of the figures in Table II that the rate of maltose formation in all of the reaction mixtures was much greater in the second half hour than in the first half hour period of the reaction. This was undoubtedly due to the fact that, as the reaction progressed and the amount of undegraded starch progressively diminished, increasingly larger quotas of the enzyme became available for reaction with the smaller dextrin molecules to form maltose.

In the second half hour a shift occurred also in the quantitative relationship between maltose and glucose, owing to competition between the maltose-forming and glucose-forming processes. In this competition the formation of maltose is increasingly favored as the dilution of the reaction mixture becomes greater. This fact indicates that maltose, at least the bulk of it, originates from larger polysaccharide molecules than does all or at least the greater part of the glucose.

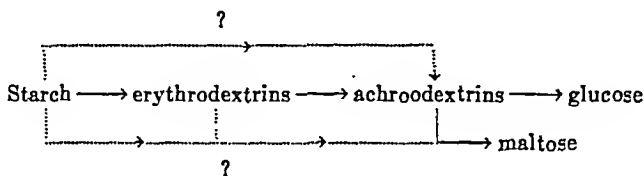
#### SUMMARY

1. The earlier finding that, besides reducing dextrins and maltose, glucose is a normal product of diastatic reactions was reaffirmed.

2. The same diastase preparation, depending upon experimental conditions, may react with the same substrate to form (1) no other sugar than non-fermentable polysaccharides, or (2) non-fermentable reducing substances plus maltose, where the latter appears as the end-product of the reaction, or (3), in addition to reducing dextrins and maltose, appreciable amounts of glucose.

3. The quantitative relationship of the three categories of reducing substances and the shifts in the rates of their formation are governed by substrate competition for the enzyme. The substrates involved are unaltered starch and dextrans of variable molecular size. In the competition for combination and reaction with diastase, preference is with the polysaccharides of larger molecular size.

4. Diastatic reactions run a course represented by the following diagram.



This work was aided by a contribution from Mr. Hugo F. Urbauer.

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# BLOOD DIASTASE IN HEALTH AND DIABETES\*

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Interest in blood diastase has greatly increased in recent years, mainly on account of its value in clinical diagnosis. Inflammatory conditions and duct occlusions of the pancreas were long known to increase the diastatic activity of blood to far above the normal level. Myers and Killian claimed that blood diastase is substantially elevated in diabetes also, and deduced a sweeping theory from their finding (1). The inadequacy of the analytical method of these authors, which was pointed out in a previous communication from this laboratory (2), impelled us to reexamine the problem.

The initial step in this work was determination of the range of variations of the blood diastase in healthy individuals. The attitude often taken in clinical studies of considering hospital patients as normal in any relation except their particular known disease was avoided. The subjects in these observations were all people with no known disease; they were recruited from the ranks of the medical, nursing, and laboratory staffs, and were scrutinized for any detectable abnormalities. In all of the normal and in many of the diabetic cases, diastase determinations were carried out with both of our analytical methods (2).

Since the diastase values were found to be scattered over a wide range, it was deemed necessary to use a fairly large number of subjects in order to obtain usable information. At the time these studies were completed some years ago, 170 subjects were examined; since then numerous additional determinations yielded results which are in harmony with the earlier findings.

The normal diastase values are graphically shown in the left half of Fig. 1, the horizontal bars representing the per cent distribution of diastatic activity at various levels. It may be noted

\* This work was aided by a contribution from Mr. Hugo F. Urbauer.

that although the spread is considerable, 80.5 per cent of all the cases fall within the range of 80 to 150. The values above 150

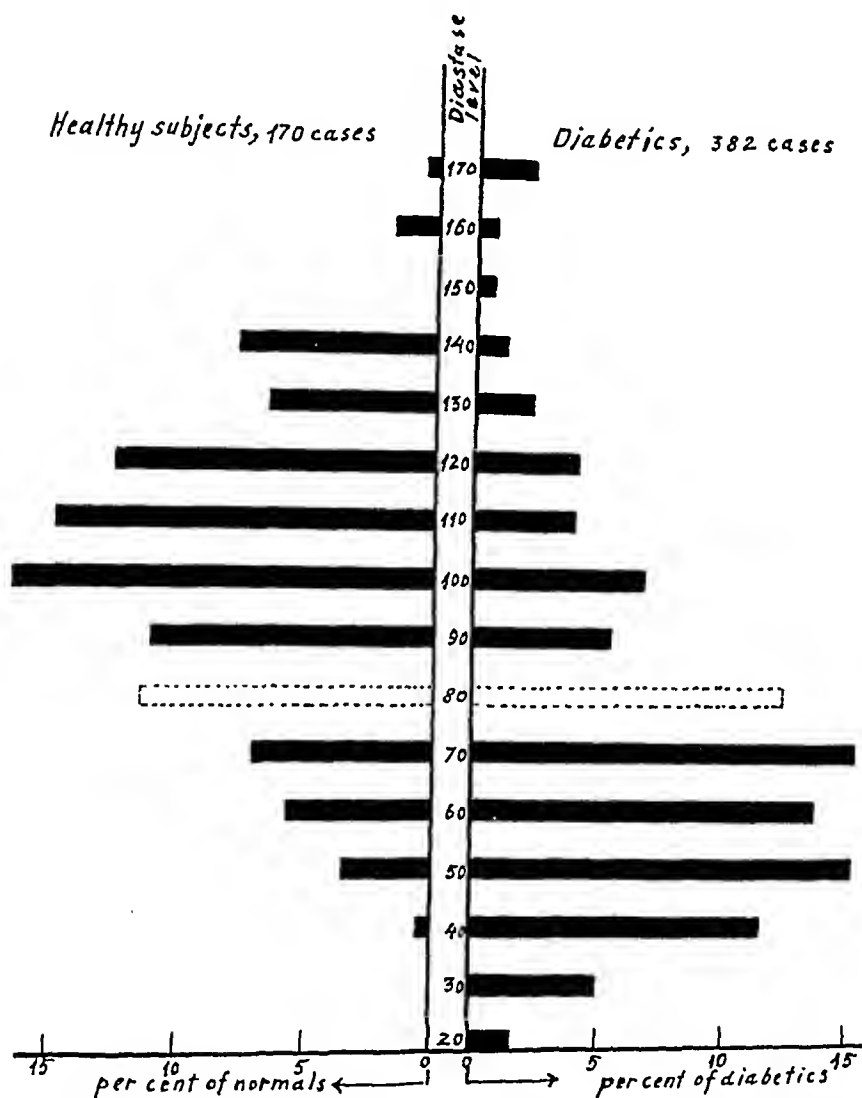


FIG. 1. Contrast between healthy and diabetic individuals in regard to the distribution of blood diastase values over the normal and subnormal range.

amount only to 2.4 per cent of the total, and none of them reaches 200. There is a greater scattering below 80, in that 13.0 per cent of the cases range from 60 to 80, and 4.1 per cent are even lower,



of the disease when the conventional, known diagnostic procedures fail to disclose any signs of abnormality. In line with this interpretation, moderately low and border line values were consistently found in those instances in which obesity resulting from overeating was a significant part of the case histories. Various degrees of fatty infiltration or degeneration of the liver in such cases are a very likely condition that may account for the depression of the diastatic activity of the blood and at the same time may play a significant part in the pathogenesis of the diabetic condition.

Definitely subnormal diastase levels were always observed in patients in ketosis, the severity of ketosis and the depression of diastatic activity showing a notable parallelism. In diabetic coma and preceding coma, diastase values as low as 20 to 30 were observed.

The results of this study tend to direct attention to the rôle of the liver in diabetes mellitus, and bring to one's mind pertinent views of well known investigators, as represented by the following remark of Krehl (6): "The behavior of the liver will always remain of great interest with respect to the various forms of diabetes. After having stood at one time in the center of interest, it is now undeservedly relegated to the background."

This work was aided by a contribution from Mr. Hugo F. Urbauer.

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# THE COLORIMETRIC ASSAY OF WEAKLY PHENOLIC KETONES, "ESTRONE," IN EXTRACTS OF HUMAN URINE\*

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Though biological methods for the determination of estrogenic hormones have the advantage of proving the presence of physiologically active material, they have recognized limitations. Callow *et al.* (1) have expressed the opinion that, "Biological assay of estrogenic activity is an unsatisfactory method of examining the mixture, consisting of estrogens and material augmenting their activity, obtainable from urine." The chemical methods of assaying human urine are, so far as we are aware, applicable only to urine of pregnant women (2-9). Any improvement in chemical assay overcoming this limitation and permitting the observation of change in endocrine function would, therefore, be an improvement over existing methods of chemical analysis.

The present paper reports a procedure for the determination of the weakly phenolic ketone (estrone) content of extracts of human urines. It is not concerned with the problem of obtaining optimal extracts. For the purposes of this study the procedure of hydrolysis and extraction recommended by Smith *et al.* (8) has been adopted. The purification procedure of Cohen and Marrian (3) has been modified to include several additional washings with 0.1 N alkali and to include a reducing agent (sodium

\* This work was supported by a grant from the Commonwealth Fund and was aided in part by grants from the Milton Fund of Harvard University and the Rockefeller Foundation.

hydrosulfite) which facilitates the removal of non-estrogenic substances from the extract. The "estrone" fraction thus obtained is further purified by the use of Girard's Reagent T which separates ketonic from non-ketonic substances. The ketonic fraction thus obtained should theoretically contain only substances which are weakly phenolic and at the same time ketonic. Thus far estrone is the only example of that type of compound which has been isolated from extracts of human urine. The assay depends upon the formation of a colored compound when estrone is coupled with diazotized dianisidine. This coupling depends upon the presence of a phenolic hydroxyl group. No color is obtained, therefore, with non-phenolic ketones such as the neutral 17-ketosteroids (androgens). Preliminary data on the "estrone" excretion determined by this procedure indicate that observed 24 hour values ranging from 0 to 600 micrograms correspond closely with the physiological status of the individuals studied.

#### *Analytical Procedure*

*Reagents*—All reagents are of c.p. grade unless otherwise specified.

Petrolatum, U.S.P., used in minimal amount on stop-cocks.

Hydrochloric acid, commercial.

Hydrochloric acid.

Ethyl ether, anesthetic or c.p. Baker's analyzed anhydrous freshly distilled in an all-glass distilling apparatus over CaO and stored in a dark bottle with bright iron wire.

10 per cent, 0.1 N and 1.0 N NaOH aqueous solutions (NaOH pellets) kept in Pyrex vessels.

0.1 N NaOH- $\text{Na}_2\text{S}_2\text{O}_4$  reagent. Prepared immediately before use by adding 50 cc. of 0.1 N NaOH solution to 5 gm. of  $\text{Na}_2\text{S}_2\text{O}_4$  (J. T. Baker Chemical Company) in a 50 cc. glass-stoppered graduate.

Approximately 0.5 N HCl (50 cc. of concentrated HCl diluted to 1 liter).

Toluene.

2 and 20 per cent  $\text{Na}_2\text{CO}_3$  solution, stored in a Pyrex container.

Litmus and Congo red paper.

Betaine hydrazide hydrochloride (Girard's Reagent T) (East-

man Kodak Company), kept in a vacuum desiccator over concentrated  $\text{H}_2\text{SO}_4$ .

Glacial acetic acid.

Concentrated sulfuric acid.

Absolute alcohol.

5 per cent sodium nitrite solution.

5 per cent urea solution.

Dianisidine dihydrochloride. Technical grade dianisidine (Eastman) is treated as follows: To 10 gm. of crude powdered dianisidine (m.p.  $134.5\text{--}136.5^\circ$ ) are added 140 cc. of distilled water and 6 cc. of concentrated  $\text{HCl}$ . The mixture is stirred well and boiled until all solid material is dissolved. 0.5 gm. of stannous chloride is added to the dark solution and boiling continued 5 minutes. To remove the slight remaining color, 1.0 gm. of norit A (Pfanstiehl) is added and the hot solution is rapidly filtered. To the hot filtrate 50 cc. of concentrated  $\text{HCl}$  are added. After cooling in an ice bath, the precipitate is filtered with the aid of suction. The crystals are washed once with 10 cc. of 95 per cent ethanol and then dried over  $\text{NaOH}$  in a vacuum desiccator until free from  $\text{HCl}$ . The resultant dianisidine dihydrochloride is colorless and has a melting point of  $283^\circ$  (decomposes).

Diazotized dianisidine. To 25 mg. of dianisidine dihydrochloride add 3 cc. of distilled water. Mix carefully until dissolved and add 0.3 cc. of concentrated  $\text{HCl}$ . Then add 0.6 cc. of freshly prepared 5 per cent sodium nitrite solution and mix thoroughly. After a period of 5 minutes, add 0.6 cc. of a 5 per cent urea solution. Mix thoroughly and let stand 5 minutes or until all the bubbles of gas have come off. The reagent is then ready to use. It is stable for about 6 days if kept in a dark brown bottle at room temperature.

*Collection, Hydrolysis, and Extraction of Urines*—Glass-stoppered containers and all-glass apparatus chemically cleaned are used throughout. The 24 hour urine sample is collected with 7 cc. of commercial  $\text{HCl}$  added as a preservative. It is kept cool during the collection period and is hydrolyzed and extracted within 12 hours after completion of the collection period. 15 volumes per cent of commercial  $\text{HCl}$  are added and the urine brought to a boil under an all-glass reflux condenser. After it has boiled exactly 10 minutes, the mixture is cooled immediately



under a stream of cold water. When cool, it is extracted four times with one-fifth the volume of ethyl ether.<sup>1</sup> The combined ether extracts are washed once with approximately 20 cc. of 20 per cent  $\text{Na}_2\text{CO}_3$  solution. The total ether extract is then evaporated to approximately 100 cc. and transferred quantitatively to a 250 cc. Pyrex separatory funnel. The final volume is adjusted to 190 cc., so that in the subsequent purification, the amount of air in the separatory funnel is not excessive.

*Isolation of Weakly Acidic and Phenolic Substances from Ether Extract*—The strongly acidic and phenolic substances are extracted from the ether by washing alternately with three 25 cc. portions of 0.1 N NaOH and two 25 cc. portions of 0.1 N NaOH-sodium hydrosulfite reagent. In the hydrosulfite-alkali extractions, the separatory funnel is tightly stoppered and shaken thoroughly for at least 3 minutes (or until the mixture is yellow in color). The ether is then washed twice with 25 cc. portions of 0.5 N HCl and three times with 25 cc. portions of water. All the washings are discarded. The remaining ether solution is evaporated approximately to dryness and the residue transferred quantitatively to a clean 250 cc. separatory funnel with four 25 cc. portions of toluene. The weakly phenolic substances are extracted from the toluene with four 25 cc. washings of 1 N NaOH.<sup>2</sup>

The combined 1 N NaOH extracts are made acid to Congo red paper (blue) with about 10 cc. of c.p. HCl and then adjusted to a pH that is acid to litmus and alkaline to Congo red by the addition of 20 per cent  $\text{Na}_2\text{CO}_3$  solution. This solution is cooled and extracted five times with 40 cc. portions of ethyl ether in a clean

<sup>1</sup> The urine may be extracted with carbon tetrachloride in a  $\frac{3}{4}$  hour period with the apparatus described by Hershberg and Wolfe (10). The carbon tetrachloride solution is evaporated to dryness and the residue transferred with 190 cc. of ethyl ether to a 250 cc. Pyrex separatory funnel. Following this the stated procedure for isolating the weakly acidic and phenolic substances is carried out.

<sup>2</sup> The total,  $\alpha$  and  $\beta$  neutral 17-ketosteroids (androgens) which remain in the toluene may be assayed colorimetrically (11) after the toluene is washed three times with water, the toluene solution evaporated to dryness, and the residue taken up in absolute alcohol. Recovery of known amounts of crystalline dehydroisoandrosterone added after hydrolysis and extraction but prior to the first 0.1 N NaOH washing has averaged 94 per cent in four experiments.

separatory funnel. The combined ether extracts are washed once with 25 cc. of 0.1 N NaOH, once with 25 cc. of 0.1 N NaOH- $\text{Na}_2\text{S}_2\text{O}_4$  reagent, shaken at least 3 minutes, once with 25 cc. of 0.1 N NaOH, twice with 25 cc. portions of 0.5 N HCl, and three times with 25 cc. portions of water. This purified ether extract is evaporated to dryness.

*Treatment with Girard's Reagent T*—The residue (light yellow or brown) is transferred quantitatively with four 1 cc. portions of absolute alcohol to a small Pyrex vessel graduated at 4 cc. and fitted with a removable all-glass reflux condenser. The final volume of the alcoholic solution should be between 4.0 and 4.2 cc. 0.5 cc. of glacial acetic acid and 0.5 gm. of Girard's Reagent T are added to the alcohol solution. The mixture is refluxed on the water bath for  $\frac{1}{2}$  hour. After cooling, the mixture is transferred with 40 cc. of ice-cold distilled water to a separatory funnel. 3 cc. of 10 per cent aqueous NaOH solution are added. The mixture is then extracted four times with 40 cc. lots of ethyl ether. These ether extracts (non-ketonic fraction) are discarded. The remaining aqueous mixture is delivered into a flask containing 1 cc. of concentrated  $\text{H}_2\text{SO}_4$  diluted with 60 cc. of distilled water. The acidified mixture is transferred to a clean separatory funnel. 40 cc. of ethyl ether are added, and the mixture is allowed to stand at room temperature for about 75 minutes. It is then extracted with the ether already added and with four additional 40 cc. lots of ether. The combined ether extract is washed once with 25 cc. of 0.1 N NaOH and three times with 25 cc. lots of water. After the final water is carefully drained from the ether, the ether is transferred to a distilling flask and evaporated to dryness. The residue (weakly phenolic ketones) is dissolved in a measured volume of absolute alcohol (usually 1 to 4 cc.).

*Colorimetric Assay*—0.3 cc. of the absolute alcoholic solution of ketones is delivered at the bottom of a 75 by 10 mm. dry test-tube. 0.75 cc. of 2 per cent sodium carbonate solution is then delivered into the tube. It is important to pipette these two solutions into the lower part of the test-tube without wetting the side walls above the level of the meniscus. 0.1 cc. of the diazotized dianisidine reagent is delivered as rapidly as possible, and the mixture is immediately mixed by stirring a few seconds with a footed glass rod. After 1 minute, 0.8 cc. of toluene is delivered

into the tube with a 1 cc. tuberculin syringe fitted with a 19 gage steel needle. The tube is stoppered with a finger and shaken vigorously for a few seconds. A blank prepared in the same way, but containing no hormone, is developed in the same manner. The tubes are stoppered with cork and centrifuged at about 2500 R.P.M. for 5 minutes. After centrifuging, the lower aqueous phase should be essentially colorless; the colored toluene layer should be clear and the color evenly distributed.

0.6 cc. aliquots of the clear supernatant toluene from the tubes containing the blank and unknown preparations are carefully transferred with the tuberculin syringe to 1 cc. absorption cells

TABLE I

*Single Sample Experiment Taken from Series Establishing Proportionality Constant between Total Crystalline Estrone Determined in Sample and Amount Shown by Galvanometer Reading*

Total estrone in sample analyzed*	Galvanometer reading	K
$\gamma$		
2.5	90	0.0183
5.0	81	0.0183
7.5	75	0.0167
10.0	65	0.0187
20.0	43	0.0184
30.0	29	0.0179
Average.....		0.0181

\* Six-eighths of this amount is read in the colorimeter; see "Analytical procedure."

of an appropriate photoelectric colorimeter.<sup>3</sup> The toluene in the cells should be clear and free from water droplets or precipitate. By means of a filter with maximum transmission at 420  $m\mu$ , range 380 to 480  $m\mu$ , the galvanometer with the absorption cell containing the blank in place is adjusted to the desired reading. This cell is then replaced by that containing the unknown sample, and the galvanometer reading of the unknown is obtained. The number of micrograms of weakly phenolic ketones (estrone) is determined by referring the galvanometer reading to a calibration

\* We have used the Evelyn microcolorimeter with an open type absorption cell of 1 cc. capacity and Filter 420.

tion curve in which galvanometer readings are plotted against known amounts of crystalline estrone (2.5 to 30.0 micrograms). Or, when the galvanometer reading of the unknown is set at 100, the micrograms of "estrone" in the sample analyzed are calculated from the equation  $C = 1/K (2 - \log G)$ , where  $K$  is the constant of proportionality determined from solutions containing known amounts of crystalline estrone (see Table I) and  $G$  is the galvanometer reading of the unknown.

### Results

Fig. 1 shows the extinction-wave-length curves of the colored compounds formed by coupling diazotized dianisidine with crystalline estrone, estradiol, and two samples of "estrone" extracted from the urines of two pregnant women. The shapes of the four curves are identical within the limits of experimental error. This identity, the absence of estradiol from the purified ketonic fraction of the extraction procedure, and the lack of any coupling of the neutral ketones with the reagent indicate that the weakly phenolic ketones extracted from human urine and assayed by this method are at least similar to crystalline estrone.

The data obtained from a single sample experiment from among those carried out in establishing our calibration curve and constant of proportionality ( $K$  in the equation above) are presented in Table I. They indicate that the crystalline estrone in amounts ranging from 2.5 to 30 micrograms per sample analyzed may be determined with an error of approximately 10 per cent. The suggested linear proportionality between the color developed and the estrone concentration has been amply confirmed by many similar analyses.

The data presented in Table II show an average recovery of 72 per cent (range 60 to 88) of crystalline estrone in pure solutions in the purification procedure of the ether extract up to treatment with Girard's Reagent T.

The data of Table III show an average recovery of 95 per cent of crystalline estrone in the ketonic fraction after treatment with Girard's Reagent T. The data of Experiment 8 show, as might be expected, that the non-ketonic estradiol does not appear in the ketonic fraction. The absence of any color in the blank analyses, Experiments 1 to 3, shows that there are no extraneous

chromogens in the ketonic fraction obtained from pure absolute alcohol. This is of importance, because small amounts of such extraneous substances may be encountered in the ketonic fraction of blank solutions treated with Girard's Reagent T according to the procedure described previously (11).

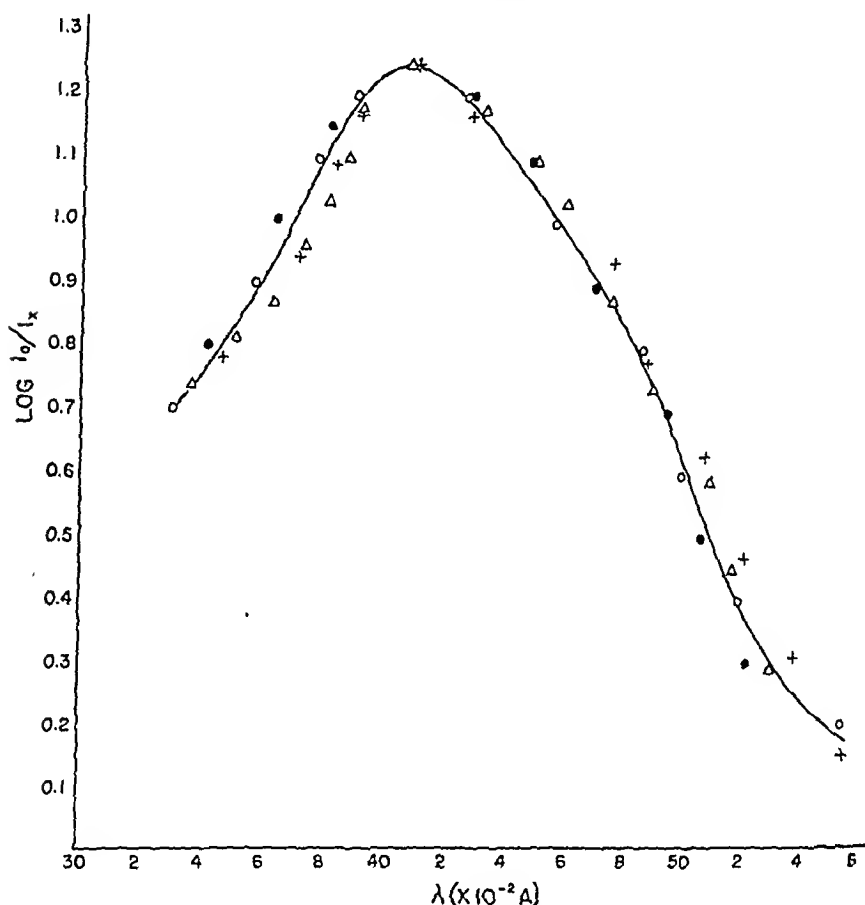


FIG. 1. Shape of the extinction-wave-length curves for reaction mixtures containing estrone, estradiol, and urinary "estrone" extracts. ● = estradiol, ○ = estrone, + = pregnancy urine Extract 1, Δ = pregnancy urine Extract 2.

The data of Table IV show the recovery of crystalline estrone added to one-half of the crude ether extracts of hydrolyzed urines from preadolescent children. The hormone was added after hydrolysis and extraction but prior to the first 0.1 N NaOH washing. The "estrone" assay of the extracts to which no crystalline estrone was added averaged 1.3 micrograms per 24 hour sample. The

recovery of estrone in the halves of the crude ether extracts containing added hormone averaged 65 per cent, with a range of from 57 to 79 per cent. This agrees with the recovery in pure solutions of crystalline estrone in the complete purification procedure (see Tables II and III) where the average recovery was 67 per cent.

TABLE II

*Recovery of Crystalline Estrone in Purification of Extracts up to Treatment with Girard's Reagent T*

Experiment No.	Theoretical (1)	Determined (2)	Ratio (2):(1)
	$\gamma$	$\gamma$	
1	25	20	0.80
2	50	33	0.66
3	50	30	0.60
4	100	66	0.66
5	100	86	0.86
6	200	146	0.73
7	200	140	0.70
8	200	154	0.77
Average.....			0.72

TABLE III

*Recovery of Estrone in Ketonic Fraction after Treatment with Girard's Reagent T*

Experiment No.	Theoretical (1)	Determined (2)	Ratio (2):(1)
	$\gamma$	$\gamma$	
1	0	0	0
2	0	0	0
3	0	0	0
4	200	179	0.90
5	200	200	1.0
6	100	94	0.94
7	25	24	0.96
8	0 + 200*	0	0

\* Estradiol.

The micrograms of weakly phenolic ketones (estrone) excreted per 24 hours in the urine of normal cyclic and pregnant women are presented in Table V. The relative order of magnitude of these excretion data corresponds with the physiological status of the subjects.

TABLE IV

*Recovery of Known Amounts of Crystalline Estrone Added to One-Half of the Crude Ether Extracts of 48 Hour Pooled Hydrolyzed Urine from Preadolescent Children*

The results are expressed as total micrograms, per 24 hour sample.

Experiment No.	Estrone added	Theoretical (1)	Determined (2)	Ratio (2):(1)
1	None		0	
	400	400	254	0.64
2	None		4	
	400	404	235	0.58
3	None		0	
	400	400	315	0.79
4	None		0	
	200	200	131	0.66
5	None		0	
	200	200	113	0.57
6	None		4	
	200	204	133	0.65

TABLE V

*Weakly Phenolic Ketones (Estrone) Excreted per 24 Hours in Urines of Normal Cyclic and Pregnant Women*

Case No.	Day of cycle or month of gestation	Weakly phenolic ketones
	days	γ
1	5	27
	11	37
	18	39
2	5	25
	11	36
	18	33
	26	50
	mos.	
3	4	420
4	7	325
5	7	600
6	7	460

## DISCUSSION

The Smith *et al.* (8) procedure has been employed for the hydrolysis and extraction of the urine samples. They have presented evidence which indicates that the procedure permits a

reasonably satisfactory recovery of estrogen. The additional washings with 0.1  $\text{N}$  NaOH and 0.1  $\text{N}$  NaOH-sodium hydrosulfite solution have been added to the Cohen and Marrian procedure (3) in order to remove substances chemically similar to quinones which are alkali-soluble when reduced but not when in the oxidized form. The evidence presented indicates that the hydrosulfite does not injure the hormones. The hydrochloric acid washings have the purpose of removing any residual hydrosulfite reagent. Girard's Reagent T serves to separate the ketonic estrogen, estrone, from the non-ketonic substances which may include estradiol and estriol.

Diazotized dianisidine was chosen as the most satisfactory color reagent, from a group of stable diazonium compounds suggested by Dr. N. A. Dahlen.<sup>4</sup> The reagent reacts to give a colored compound with phenolic substances. Because the neutral 17-ketosteroids are not phenolic, they give no color with the reagent. This characteristic makes dianisidine more suitable than *m*-dinitrobenzene which reacts to give a color with any 17-ketosteroid. The pH range at which diazotization of estrone will take place has been studied. It was found that the intensity of color given by a standard amount of the hormone varied slightly but consistently over a pH range of 7 to 10. The amount of sodium carbonate recommended gives a pH between 8 and 8.5, which is the range of optimal color development. A study was made of the other variables (amount of water, alcohol, reagent, and toluene), and their final proportions were selected to satisfy the optimal conditions.

The loss of estrone encountered in the purification procedure is greater than could be desired, but being reasonably constant does not seriously interfere with the significance of the procedure.

The data obtained on various types of individuals show the daily output of weakly phenolic ketones to be very low in normal preadolescent children, somewhat higher in young adult women, and much higher in pregnant women. In the absence of biological assays, it cannot be stated with certainty at present that the substance measured is estrone. However, as far as we are aware, estrone is the only weakly phenolic ketone known to be present in

<sup>4</sup> Staff member, E. I. du Pont de Nemours and Company, Inc., Wilmington.



extracts of hydrolyzed human urine. It is clear that the values reported here should be compared only with colorimetric or biological assays in which estrone has been separated from the other estrogens. Such a comparison has been made with unpublished data of Smith.<sup>5</sup> According to her, the values reported here are within the limits of those obtained on subjects of corresponding physiological status by biological assay of the ketonic portion of the estrone fraction (8).

#### SUMMARY

The present paper reports a method for the determination of the weakly phenolic ketone (estrone) content of the 24 hour urine extracts from patients of various ages. The relative "estrone" values obtained correspond closely to the physiological status of the individuals studied.

The authors are indebted to Dr. L. F. Fieser who made many valuable suggestions during the course of this investigation. Dr. E. Schwenck, of the Schering Corporation, generously supplied the crystalline estrone and estradiol used herein.

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## STUDIES ON THE CHEMISTRY OF THE FATTY ACIDS

### VI. THE APPLICATION OF CRYSTALLIZATION METHODS TO THE ISOLATION OF ARACHIDONIC ACID, WITH A COMPARISON OF THE PROPERTIES OF THIS ACID PREPARED BY CRYSTALLIZATION AND BY DEBROMINATION. OBSERVATIONS ON THE STRUCTURE OF ARACHIDONIC ACID

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In previous work from this laboratory methods have been developed for the direct isolation of unsaturated acids by fractional crystallization at low temperature. By these methods it has been possible to prepare oleic acid of high purity from olive oil (1), linoleic acid 90 to 94 per cent pure from cottonseed and corn oils (2, 3), and 85 to 88 per cent linolenic acid (4) from linseed and perilla oils. These methods have been especially valuable, because for the first time it has been possible to prepare the latter two acids by a method other than the classic bromination-debromination procedure. The yields by the crystallization methods are high but the purity of the product is not so satisfactory as that obtained by reduction of the bromides.

One of the principal objectives in the present investigation was to apply the crystallization procedures to the isolation of arachidonic acid,  $C_{20}H_{32}O_2$ . In a previous investigation Ault and Brown (5) described the preparation of methyl arachidonate from the fatty acids of suprarenal phosphatide by three procedures; namely, direct fractional distillation of the methyl esters, the lithium soap-acetone method of Tsujimoto, and reduction of methyl octabromoarachidate.

In the present paper it is shown that methyl arachidonate of 70 to 75 per cent purity can be prepared by fractional crystallization of the methyl esters of suprarenal phosphatides from acetone. Higher purity was not attainable by these methods, because

impure methyl arachidonate is liquid even at  $-80^{\circ}$ . However, by efficient fractional distillation of the 75 per cent product the purity was raised to 95 per cent. As in the cases of linoleic and linolenic acids, it is possible to obtain much higher yields of less pure material by the crystallization methods. In the debromination procedure about three-fourths to four-fifths of the arachidonic acid is "lost" in the liquid bromides.

The constants of three specimens of methyl arachidonate, prepared by the debromination method, are compared with five specimens prepared by crystallization-distillation. The former specimens show appreciably lower polybromide numbers than the latter, prepared by purely physical methods. It is concluded that methyl arachidonate obtained by debromination is essentially the same compound as the ester prepared by crystallization, but the former does contain small amounts of an isomeric ester which gives a low polybromide number.

Previous observations on the structure of arachidonic acid have been confined to analyses of the iodine number and mean molecular weight (by titration), bromine content of the methyl ester octabromide, and molecular weight and melting point of the reduced acid, arachidic. These constants definitely established the formula,  $C_{20}H_{32}O_2$ , with four double bonds and a straight carbon chain. In this report the straight chain is further confirmed by constants not only on the reduced acid but also on the methyl and ethyl esters of the reduced acid, all of which are in agreement with the constants of synthetic *n*-eicosanoic acid and its esters. The position of the double bonds in arachidonic acid has not been previously determined. Toyama and Tsuchiya (6) have isolated an eicosatetrenoic acid from sardine oil and have ascertained the position of its double bonds to be  $\Delta$ -4-8-12-16. This structure was deduced from the products of ozonolysis which were exclusively succinic and butyric acids. In the present work the almost quantitative yields of products, by the ozonolysis and permanganate methods, obtained by the above investigators, were not obtained. From our data, it seems likely that one double bond is in the  $\Delta$ -6 and another is in the  $\Delta$ -18 position. The evidence is against the presence of double bonds in the  $\Delta$ -9-12 positions as in linoleic and linolenic acids. There is some evidence based on the diene number that the methyl arachidonate prepared

by debromination contains at least 5 per cent of conjugated bonds. This fact would support the view that the ester is a mixture of at least two compounds, one of which contains conjugated unsaturation. The thiocyanogen number of methyl arachidonate is one-half the iodine number.

Although our data on the position of the double bonds are far from presenting a complete story, we include them here as a preliminary report, in view of the biological interest in arachidonic acid and because it does not seem possible to complete this work for some time.

#### EXPERIMENTAL

*Preparation of Esters*—The raw material used in this work was the phosphatide fraction of the lipids of beef suprarenals, the fatty acids of which have been shown to contain about 20 per cent of arachidonic acid (5), which was kindly furnished us by Dr. Oliver Kamm of Parke, Davis and Company. The phosphatides were converted into methyl esters by direct alcoholysis as described by Shinowara and Brown (7).

*Crystallization Experiments*—We have carried out a large number of crystallization experiments, starting with both acids and methyl esters. The fatty acids, having higher melting points, are more adapted to crystallization procedures, but it is more advantageous to use the esters, because the resultant products may be directly distilled without danger of serious alteration. It is to be noted that, unlike previous work with oleic, linoleic, and linolenic acids, crystallization procedures for arachidonic acid or its methyl ester are limited by the fact that the desired product is a liquid at  $-80^{\circ}$  and thus is always carried over into the final filtrate. The following general procedure will result in a product containing 70 to 75 per cent methyl arachidonate.

A 7 to 10 per cent solution of the esters in acetone is allowed to stand overnight at  $-20^{\circ}$ . The crystals, containing most of the saturated esters, are filtered off with suction. If desired, they may be again dissolved and recrystallized and the two filtrates combined. The filtrate is cooled in a bath of dry ice and methyl alcohol to  $-70^{\circ}$ . The crystals, containing most of the methyl oleate, are again filtered with suction in the  $-20^{\circ}$  room as rapidly as possible. The filtrate, containing esters which are 50 to 60

per cent methyl arachidonate, is concentrated to about one-fourth its original volume and again cooled to  $-70^{\circ}$  and filtered. The final filtrate contains 70 to 75 per cent arachidonate. So far we have been unable to obtain a product of higher purity by crystallization methods alone, but by fractional distillation it is not difficult to increase the purity to 90 to 95 per cent.

In the work which follows there are described briefly the results of four crystallization-distillation experiments and in addition two preparations of methyl arachidonate by the debromination procedure.

*Experiment I*—800 gm. of esters in 12 liters of acetone were cooled to  $-20^{\circ}$ , filtered, and the precipitate recrystallized from 8 liters of acetone at the same temperature. Yield, 325 gm. of crystals; iodine number, 4.2. The combined filtrates were reduced in volume to 10 liters and cooled to  $-65^{\circ}$ . An additional 243 gm. of precipitate were filtered off. The esters of the filtrate contained 56 per cent methyl arachidonate. One crystallization of 175 gm. of these esters in 800 cc. of acetone at  $-75^{\circ}$  gave 100 gm. of 70 per cent product. This was distilled, the fraction coming over at  $190-205^{\circ}$  at 3 mm. amounting to 20 gm. (84.6 per cent methyl arachidonate). It is further described in Table I.

*Experiment II*—2 kilos of esters (polybromide number, 20.2) were made up to 24 liters in acetone and directly cooled to  $-70^{\circ}$ . The crystals were recrystallized from 16 liters of acetone at the same temperature. The combined filtrates contained 660 gm. (iodine number, 192.5; polybromide number, 42). 640 gm. of this product were cooled to  $-75^{\circ}$  in 3000 cc. of acetone. Yield, 325 gm. of crystals; iodine number, 168; polybromide number, 28. Yield, 315 gm. of filtrate esters; iodine number, 260. Fractional distillation of 270 gm. of this material at 1 mm. from a Claisen flask with a 25 cm. indented side arm gave three fractions as follows:

Fraction No.	B.p.	Weight	I No.	Polybromide No.
	$^{\circ}\text{C.}$	gm.		
1	Below 175	170	229.1	40.1
2	175-195	86	296.2	79.2
3	Above 195	8	306.2	86.2

Fractions 2 and 3, containing 91.5 and 99.5 per cent methyl arachidonate respectively, are described further in Table I.

*Experiment III*—From several crystallization experiments, not described here, there was available a considerable quantity of

TABLE I

*Analytical Data on Methyl Arachidonate Prepared by Different Methods*

Specimen	Mean mol. wt.	$n_D^{20}$	Iodine No.	Polybromide No.		Thio-cyano-gen No.	Per cent purity from	
					On 100 per cent basis		Iodine No.	Poly-bromide No.
1. Crystallization-distillation, Experiment I.	313.7		289.3	84.6	97.7		87.5	97.8
2. Same, Experiment II, Fraction 2.....	315.2	1.4800	296.2	79.2	87.0		91.0	91.5
3. Same, Experiment II, Fraction 3.....	317.7	1.4845	306.2	86.2	90.7		95.0	99.5
4. Same, Experiment III.....	316.5	1.4812	300.3	80.0	87.0		92.0	92.0
5. Same, Experiment IV.....	317.2	1.4815	306.0	86.3	91.4	159.0	94.4	99.8
6. Reduction, Experiment V...	318.0	1.4824	319.0	86.3	86.3	161.1	100.0	100.0
7. Reduction, Experiment VI..	315.8	1.4823	312.8	84.1	86.3		97.5	97.5
8. Reduction of bromides*.....	318.8	1.4723	316.9	86.5	86.6		99.5	100.0
9. Lithium soap-acetone*.....	319.2		286.2	76.2	88.0		86.0	88.2
10. Fractional distillation*.....	311.5		261.8	74.4	99.0		75.6	86.0
Theory.....	318.0		319.0			159.5†		

\* From Ault and Brown (5).

† For two double bonds.

75 per cent methyl arachidonate. 370 gm. of this material were fractionated once through a special 25 mm. vacuum-jacketed column, 75 cm. long. The fraction coming over at 170–172° at 0.5 mm., amounting to 145 gm., gave an iodine number of 300.3 (see Table I).

*Experiment IV*—There were available 135 gm. of esters with an iodine number of 300.6 and 70 gm. with an iodine number of 302.4. These were combined and redistilled in the column described in Experiment III. The 157.5–160° fraction, 145 gm., gave an iodine number of 306.0 (see Table I).

*Experiment V*—2330 gm. of distilled methyl esters obtained from various intermediate crystallization fractions and containing 26.5 per cent methyl arachidonate as calculated from the polybromide number were dissolved in 11 liters of ether and brominated at 0°. The octabromides were washed six times by decantation with ether at 0°; yield, 516 gm.; m.p., 228.5–229.5° (uncorrected); bromine, 66.6 per cent (theory, 66.7%).

410 gm. of the bromides were suspended in 1.5 liters of boiling methanol and reduced with zinc for 24 hours. The yield of the distilled product was 92 gm. (70 per cent of theory) (see Table I).

*Experiment VI*—52 gm. of methyl arachidonate were obtained by the bromination-debromination method from 1750 gm. of original esters (polybromide number, 18.2) (see Table I).

#### DISCUSSION

In Table I the analytical constants of five of the crystallization-distillation preparations are compared with those of methyl arachidonate obtained by debromination and by other methods.

Purities in Table I are calculated from the iodine numbers, assuming the specimens to be mixtures of methyl arachidonate and oleate. Since it is possible that the impurity is not entirely methyl oleate but may consist in part of some saturated esters, we believe the per cent purity as calculated from the iodine number is low. Purity was calculated from the polybromide number by the method of Ault and Brown (5). The data are interesting from a number of view-points.

*Yield*—By the bromination method it is possible to prepare about 80 to 90 gm. of pure ester from 1850 gm. of phosphatide esters. This represents less than one-fourth the total amount present, the remainder being lost principally as soluble octabromides. In contrast to these results in Experiment I there were obtained 100 gm. of 70 per cent methyl arachidonate from 800 gm. of esters and, in Experiment II, 315 gm. of 75 per cent product from 2 kilos of the esters. In addition to these products

it would not be difficult to obtain from the discarded fractions additional amounts of the desired ester by repeating the crystallization-distillation procedures. An efficient application of these methods therefore would result in the isolation of something approaching the theoretical amount of methyl arachidonate present. On the other hand the purity could not equal that of the debromination product.

*Identity of Products Obtained by the Two Procedures*—The purities as calculated from the iodine and polybromide numbers show satisfactory agreement in all specimens except Nos. 1 and 10. Also, when the per cent purity, calculated from the iodine number, is used to correct the polybromide number to 100 per cent purity, the results show an average of 90.7 for the five crystallization specimens and 86.4 for the three reduction specimens of methyl arachidonate. We believe that these are significant but not fundamental differences, which may be explained as follows: The arachidonic acid in the original esters, and probably also in the original phosphatides, is a single acid which is the product isolated by the physical methods employed, crystallization and distillation. The ester obtained by bromide reduction, on the other hand, is a mixture of this original compound with small amounts of isomeric arachidonic acids which give a lower or possibly a zero polybromide number. Assuming the impurity to be a compound giving a zero polybromide number, it must be present to the amount of about 5 per cent to account for the difference noted. We have obtained similar results and have drawn similar conclusions in work in this laboratory on linoleic and linolenic acids. Apparently, debromination of the several bromides of the unsaturated acids is accompanied by isomerization, either of the *cis-trans* or double bond position type.

*Thiocyanogen Number*—The thiocyanogen numbers of the two specimens tested were 159.0 and 161.1 (theory, 159.5 for two double bonds). Thiocyanogen reacts practically quantitatively with two of the four double bonds of arachidonic acid (25°).

#### *Preliminary Observations on Structure of Arachidonic Acid*

Previous work on the structure of arachidonic acid has shown that it is a straight chain eicosatetrenoic acid,  $C_{20}H_{32}O_2$ . In the present work further evidence for the straight chain structure



is presented; also preliminary attempts to locate the position of the double bonds are described.

*Hydrogenation of Methyl Arachidonate*—Methyl arachidonate (Specimen 5, Table I) was hydrogenated in a Burgess-Parr apparatus. From the resulting methyl arachidate the free acid and the ethyl ester were prepared. Molecular weights and melting points are given in Table II.

*Diene Number*—For the determination of the diene number, which is a measure of conjugated double bonds, the authors are indebted to Dr. K. S. Markley of the United States Regional Soybean Industrial Products Laboratory, Urbana, Illinois. The diene numbers of methyl arachidonate for the crystallization Specimen 5 and the debromination Specimen 6 were 9.1 and 7.9 respectively. Dr. Markley interprets these numbers to indicate

TABLE II

*Analytical Data on Arachidic Acid and Its Methyl and Ethyl Esters, Prepared from Methyl Arachidonate*

	Arachidic acid	Methyl ester	Ethyl ester
Mol. wt., theory.....	312.3	326.3	340.3
" " found.....	312.6	326.7	340.1
M.p., °C.....	76.3-77.0	46-47	42.0-42.1
" n-eicosanoic acid (8), °C.	76 -77	46-47	41.5-42.5
" arachidic acid (9), °C...	77	54.5	50

that about 5 per cent of the double bonds in these products is conjugated. This may mean that the ester is a mixture of two isomeric acids, or that under the conditions of the diene number determination part of the ester rearranges into a conjugated configuration.

*Ozonolysis and Acetone-Permanganate Oxidation*—Attempts to locate the position of the double bonds in methyl arachidonate were confined to six ozonolysis and three acetone-permanganate oxidations. This latter method as described by Collins and Hilditch (10) was successfully used by Haworth (11) for determining the structure of linoleic acid. The almost theoretical yields of fragments obtained by Toyama and Tsuchiya (6) for sardine oil eicosatetrenoic acid were not obtained in any of our experiments. These investigators isolated butyric and succinic

acids in quantitative amounts. Since our work is inconclusive, we will report briefly only the facts which seem at present justified by our data.

1. Acetaldehyde was identified in two ozonolysis experiments. The volatile decomposition products, which were collected in cold water, gave a strongly positive Schiff's test; the 2,4-dinitrophenylhydrazone and the *p*-nitrophenylhydrazone melted at  $164^{\circ}$  and  $126.5^{\circ}$  respectively. The melting points of the pure acetaldehyde derivatives are  $164^{\circ}$  and  $127.5^{\circ}$ . The fragment  $\text{CH}_3\text{—CH=}$  is therefore present, indicating a  $\Delta$ -18 double bond.

2. The  $\text{=CH—CH}_2\text{—CH=}$  fragment is probably not present. Decomposition of the ozonide with hot  $\text{H}_2\text{O}_2$  solution failed to produce more than traces of  $\text{CO}_2$ . If the above 3-C fragment had been produced,  $\text{CO}_2$  would have resulted from decomposition of malonic acid.

3. Oxalic acid which might have originated from decomposition of malonic acid or from the  $\text{=CH—CH=}$  fragment could not be isolated although careful search was made.

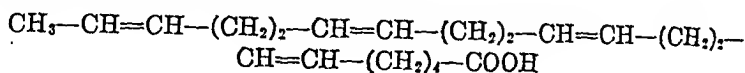
4. Azelaic acid, resulting from the  $\text{=CH(CH}_2)_7\text{CH=}$  or  $\text{=CH—(CH}_2)_7\text{—COOH}$  fragments, was not found although it was easily isolated in control experiments on oleic and linoleic acids. It is concluded that the first double bond is closer to the carboxyl than the  $\Delta$ -9 position.

5. Succinic acid was definitely identified in one ozonolysis experiment following  $\text{H}_2\text{O}_2$  oxidation. The acid melted at  $185^{\circ}$  (m.p. of succinic acid,  $189\text{--}190^{\circ}$ ). The barium salt contained 54.4 per cent Ba (theory, 54.2 per cent). Either fragment  $\text{=CH—(CH}_2)_2\text{—CH=}$  or  $\text{=CH—(CH}_2)_2\text{—COOH}$  is therefore present.

6. In two experiments adipic acid,  $\text{C}_6\text{H}_{10}\text{O}_4$ , was definitely isolated and identified; m.p.,  $154^{\circ}$  (m.p. of adipic acid,  $151\text{--}153^{\circ}$ ); neutralization equivalent, 69.9 (calculated 73.0).

As a result of this work it seems that the formula must be represented by one  $\text{CH}_3\text{—CH=}$ , three  $\text{=CH—(CH}_2)_2\text{—CH=}$ , and one  $\text{=CH—(CH}_2)_4\text{—CH=}$  fragments. We favor  $\Delta$ -6 as the position of the first double bond, because arachidonic acid gives a theoretical iodine number. It would appear that a  $\Delta$ -4 double bond would add  $\text{IBr}$  or  $\text{ICl}$  slowly. We suggest, therefore,

that the arachidonic acid occurring in adrenal phosphatides is  $\Delta$ -6-10-14-18-eicosatetrenoic acid.



We feel, however, that until we can isolate nearly the theoretical yield of products from a single experiment the above formula must be considered as tentative. It is to be observed that none of the double bonds is in the interesting 9-12 position as in linoleic and linolenic acids, the other two essential fatty acids. This work is being continued.

#### SUMMARY

1. Crystallization procedures at low temperature have been applied to the methyl esters of adrenal phosphatides. The methyl arachidonate, contained therein, has been concentrated to 70 to 75 per cent by these procedures.

2. Five preparations of methyl arachidonate, 87.5 to 95 per cent pure, prepared by crystallization and distillation have been analyzed and compared with three purer specimens prepared by reduction of methyl octabromoarachidate.

3. The polybromide numbers of the crystallization-distillation specimens averaged 90.7 as compared to 86.3 for the debromination specimens. The significance of these results is discussed.

4. Preliminary data on the structure of arachidonic acid are reported and a tentative structure proposed.

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# THE EFFECT OF ASCORBIC ACID\* ON IMINAZOLE RINGS AND ALLIED SUBSTANCES IN VIVO

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The decomposition of nitrogenous compounds (iminazoles, purines, amino acids, etc.) *in vitro* by ascorbic acid has been extensively investigated by Edlbacher and von Segesser (1), and to a lesser degree by Holtz and Triem (2). In all cases, the compounds under investigation were dissolved in phosphate buffer together with stated amounts of ascorbic acid. To facilitate the decomposition and to aid in the collection of the ammonia liberated, a stream of oxygen was passed through the solution, the temperature remaining constant. By determining the amount of ammonia evolved during the reaction, the decomposition of the nitrogenous substances was measured.

In this paper an attempt to study the decomposition of iminazoles and purines by ascorbic acid *in vivo* is described. Because of the limitations of specific and sensitive chemical tests for these compounds, only thioneine, uric acid, and histamine were studied. Uric acid was estimated by Folin's procedure (3), thioneine by the method of Hunter (4). Histamine was measured by its hormone-like action on the HCl in the gastric secretion, on the assumption that histamine is the activator of HCl in the gastric juice. Urea nitrogen was determined because of the possibility of formation of ammonia, which would be converted to urea nitrogen *in vivo*.

## EXPERIMENTAL

*In Vitro*—Since neither group of investigators had studied the action of ascorbic acid on thioneine *in vitro*, it was deemed neces-

\* The ascorbic acid used in these experiments was generously supplied by E. R. Squibb and Sons and in part by Hoffmann-La Roche and Company, A. G.

sary to perform this experiment prior to any *in vivo* studies. Using a technique similar to that of Edlbacher and von Segesser, we observed that at the end of 9 hours 14.3 mg. of N were evolved as ammonia from 100 mg. of thioneine. The technique of Holtz and Triem gave results in the same order of magnitude, but the decomposition was much slower. There was little change in the decomposition after the 9th hour, although the reaction was kept going for 24 hours.

*Experiments on Animals*—Rabbits of both sexes weighing 2.5 to 3.0 kilos were used. One group received 50 mg. of ascorbic acid per kilo, in 5 cc. of water, intravenously. 0.2 mg. of iron as

TABLE I

*Effect of Ascorbic Acid on Blood Thioneine of Rabbits and Guinea Pigs*

• The values are given in mg. per cent.

No. of animals	Before administration	After administration					
		½ hr.	½ hr.	2 hrs.	4 hrs.	8 hrs.	24 hrs.
Rabbits							
21*	7.3	7.8	7.9	7.6	7.0	7.3	8.2
16†	7.9	8.2	7.9	8.5	7.4	7.1	8.1
Guinea pigs							
28*	8.7	9.3	8.2	8.3	8.9	9.4	9.8
17‡	8.1	8.1	8.8	8.0	8.6	9.0	8.3

\* Ascorbic acid administered.

† Ascorbic acid + 0.2 mg. of iron (as ferric ammonium citrate).

‡ Ascorbic acid + 0.1 mg. of iron (as ferric ammonium citrate).

ferric ammonium citrate was given to the other group in addition to the ascorbic acid. The solutions were prepared just before use and sterilized by Berkefeld filtration. All food was withheld from the animals 7 hours before the experiment. Only water and excelsior were in the cages during the 34 hour experimental period. Blood was obtained by the intracardial route and was divided into three parts. The portion used for thioneine determinations was deproteinized with tungstomolybdic acid according to the method of Benedict and Newton (5). The portion for uric acid studies was freed of protein by tungstic acid. The remaining blood was used for urea nitrogen determinations (6). This procedure was used throughout the entire experiment. As

determined by the Hunter procedure, there was no great variation in the blood thioneine of the individual animals, so only the average group figures are presented in Table I.

Guinea pigs of both sexes, weighing 275 to 375 gm., were kept under the same environmental conditions as the rabbits. They were divided into two groups, one of which received 25 mg. of ascorbic acid in 2 cc. of sterile water intraperitoneally, and the other an equal amount of the acid plus 0.1 mg. of iron. Nineteen animals died during the course of the experiment owing to numerous bleedings. Urea nitrogen and uric acid were not determined in either the guinea pigs or rabbits because of the difficulty encountered in obtaining sufficient blood. As in the previous experiment, there was but slight variation in the findings from animal to animal and, consequently, only the average figures are presented in Table I.

TABLE II  
*Effect of Ascorbic Acid on Blood Components and Gastric HCl in Humans*

Time, hrs.....	0	$\frac{1}{2}$	1	3	5	24	
Blood thioneine, mg. %...	5.2	5.6	5.7	5.0	5.9	5.4	
" urea N, mg. %.....	12.0	12.2	11.5	13.2	12.0	13.0	
" uric acid, mg. %....	2.4	2.4	3.0	2.9	2.6	2.5	
Time, hrs.....	0	$\frac{1}{12}$	$\frac{1}{2}$	$\frac{1}{2}$	2*	2 $\frac{1}{2}$	4
Gastric free HCl, units....	17	24	30	25	29	59	21
" total " " ....	28	31	39	37	38	70	30

\* 1 mg. of histamine hydrochloride was administered intramuscularly.

*Experiments on Humans*—The fourteen subjects selected for the investigation were all males between the ages of 19 and 35 years. The Rehfuß tube was passed into the stomach of the fasting subject. Specimens of gastric juice and blood were taken before and at stated intervals after the intravenous administration of ascorbic acid. At the end of a 2 hour period, 1 mg. of histamine hydrochloride was given parenterally to determine whether its effect would be modified by the previously administered ascorbic acid. Since Potter and Franke (7) demonstrated the exogenous source of thioneine in animals, foods low in thioneine precursors were ingested between the 5 and 24 hour periods.

The average figures in Table II indicate that the action of histamine was not modified. HCl is reported in the customary units.

Seven members of the original group of fourteen men were given 2.5 mg. of iron as ferric ammonium citrate in addition to the ascorbic acid. The results were of the same order of magnitude as with subjects not receiving any intravenous iron and are therefore omitted. As measured by the gastric acidity, histamine hydrochloride parenterally administered was unaffected in action.

#### DISCUSSION

It appears from the data that comparatively large amounts of ascorbic acid injected intravenously in man and rabbit, and intraperitoneally in the guinea pig, leave the iminazole ring of thioneine and the purine ring of blood uric acid untouched. If any appreciable deamination took place in the numerous nitrogenous compounds in the blood, detectable changes above those due to technique would have been observed in the urea nitrogen determinations. No such changes were found.

We wish to thank Dr. Harold Fink, pathologist, and Mr. H. C. McCall for helping to make this work possible.

#### SUMMARY

The iminazole ring of thioneine is split hydrolytically, *in vitro*, by ascorbic acid.

Blood thioneine of guinea pigs, man, and rabbit is unaffected by ascorbic acid *in vivo*. Blood uric acid in man is also unaffected.

The iminazole ring of histamine is probably untouched by ascorbic acid *in vivo*, as measured by its hormone-like effect on the gastric secretion of HCl.

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# ELECTROLYTE AND WATER EQUILIBRIA IN THE DOG

## I. EQUILIBRIA IN THE BLOOD IN ADRENAL INSUFFICIENCY

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Many workers have been interested in determining the electrolyte and water equilibria of the body in normal individuals and also in observing the mechanisms of adjustment following disturbances encountered in disease or following disturbances produced by various experimental procedures. In 1932 Loeb (1) made the important observation that a marked lowering of the serum sodium occurs in the crises of Addison's disease. A short time later Loeb, Atchley, Benedict, and Leland (2) and Harrop and his coworkers (3, 4) observed the same change following double adrenalectomy in dogs and found it to be associated with a negative sodium balance. Among other electrolyte and water disturbances noted were decreases in serum chloride and bicarbonate, an elevation of serum potassium, and hemoconcentration. Subsequent to this work numerous publications have appeared dealing with the influence of the adrenal cortex on various phases of the metabolism of salt and water of the body.

The previous studies on dogs have indicated that rather marked electrolyte and water disturbances of the blood may be encountered in adrenal insufficiency. This fact aroused our interest in determining whether or not the physicochemical laws which have been established for normal conditions (5) also held under such circumstances. Consequently the experiments included in this paper were undertaken for the following purposes: (1) to obtain further information regarding the extent of the electrolyte and water changes of the blood of dogs in adrenal insufficiency; (2) to observe whether the distribution of electrolytes between serum



and cells is in accordance with the Gibbs-Donnan law; and (3) to observe the changes in the equilibria between serum and cells of dogs in adrenal insufficiency following the injection of cortical extract alone.

To conserve space only the literature dealing with studies on dogs or literature having a direct bearing upon the present problems will be cited.

### *Methods and Calculations*

Adult dogs were used in this study and were placed on a constant diet for at least 2 weeks before the experiments were undertaken. Aside from four dogs, which received Purina Dog Chow, the diet (high in sodium, low in potassium) suggested by Nilson (6) was employed. The control blood was withdrawn under oil from the femoral artery 24 hours after the last feeding and placed under oil in two special centrifuge tubes containing heparin to prevent clotting. The blood in the one tube served for the whole blood analyses. The second tube was immediately centrifuged for 10 minutes and the larger part of the serum was withdrawn and stored under oil for analyses. After the remaining serum had been removed and discarded, the tube was centrifuged again for 1 hour at a high rate. The contents of the latter served for the cell analyses, after any remaining serum had been carefully withdrawn.

The adrenals were removed in two stages and the animals were kept in a normal condition for a number of days following the second operation with the aid of cortical extract.<sup>1</sup> The dogs were allowed to regress into insufficiency by discontinuing the extract and by reducing the sodium chloride intake (and in most instances by also including 0.5 gm. of  $\text{KH}_2\text{PO}_4$  daily in the diet). The experimental arterial blood was obtained from animals showing rather marked insufficiency and was treated as outlined above. A brief description of the experiments in which animals in adrenal insufficiency were treated with cortical extract will be given later.

The serum was analyzed for  $\text{pH}$ , water, chloride, carbon dioxide content, total protein, sodium, and potassium. The whole blood was analyzed for oxygen and carbon dioxide content, oxygen

<sup>1</sup> The authors are indebted to Parke, Davis and Company and to The Upjohn Company for the cortical extract employed in these experiments and those reported in Paper II (7).

capacity, cell volume, water, chloride, sodium, and potassium. The cells were analyzed for water, chloride, sodium, and potassium.

For the sodium and potassium determinations weighed samples (between 5 and 6 gm.) were dried to constant weight at a temperature of 105–110°, and ashed in the same manner as described by Cullen and Wilkins (8) for tissues. After treatment in the muffle furnace, the ash was dissolved in hot water, and the volume brought to 25 cc. Following filtration, sufficient solution was obtained to make duplicate determinations of sodium by the Butler and Tut-hill (9) method and of potassium by the cobaltinitrite technique (10). All the other analyses were performed in duplicate in essentially the same manner as described elsewhere (11). Except for a relatively few instances, the pH<sub>i</sub> was determined by means of the hydrogen electrode at 38°, and, when it was necessary to employ the colorimetric method, a colorimetric correction of 0.30 pH was used (12).

The bicarbonate content of the cells was calculated indirectly from the whole blood and serum values and the cell volume. The chloride, sodium, and potassium contents of the cells were obtained both by direct and indirect analysis. With either method the cell chloride and the cell sodium concentrations usually agreed very well, but when any significant deviation was encountered the value for the direct determination was recorded. In the case of the cell potassium the agreement was not so consistent and the discrepancy was not entirely regular. Consequently, the values for cell potassium which are reported represent averages of the two determinations in each instance. The base bound by the serum protein was calculated by the equation of Van Slyke, Hastings, Hiller, and Sendroy (13) and the base bound by the cell hemoglobin was calculated by the equation of Van Slyke, Wu, and McLean (5). The distribution ratios  $r_{Cl}$  and  $r_{HCO_3}$  were corrected for incomplete oxygenation of the blood as described by Hastings, Sendroy, McIntosh, and Van Slyke (14). The latter represented only a small correction, since arterial blood was employed.

#### EXPERIMENTAL

*Electrolyte and Water Distribution in Normal Blood*—A summary of the data obtained from the analysis of the serum and cells of

twenty-four normal dogs is given in Table I. The acid-base equilibria of the two phases are on the whole similar to those reported by Dill, Edwards, Florkin, and Campbell (15). The range for the serum sodium is in agreement with that reported by Hastings and Eichelberger (16). Yannet, Darrow, and Cary (17) found the mean values for the sodium of serum and cells in

TABLE I  
*Electrolyte and Water Content of Serum and Cells of Twenty-Four Normal Dogs*

	Serum				Erythrocytes			
	Mean	Maximum	Minimum	$\sigma$	Mean	Maximum	Minimum	$\sigma$
Water*	917.6	926	912	3.6	652.8	661	643	5.0
pH <sub>s</sub>	7.42	7.5	7.3	0.06				
BCl†	117.2	121	114	2.2	80.5	89	76	2.7
BHCO <sub>3</sub> †	23.1	28	18	2.4	18.0	21	15	1.8
BPr†	16.3	19	14	1.1	63†	77	49	7.6
Determined acid†	156.6	161	153	2.4	162.2†	176	143	7.8
Na†	155.9	160	152	2.2	145.0	152	135	4.3
K†	3.5	5	2	0.5	8.9†	12	6	1.1
Determined base†	159.4	164	156	2.2	153.8†	159	143	4.6
Base minus acid†	2.8	10	-3	3.1	-8.4†	-22	14	9.2
Protein§	6.3	7	5	0.3				
Cell volume					49.4	60	32	4.6
rCl					0.682	0.73	0.66	0.016
rHCO <sub>3</sub>					0.770	0.85	0.71	0.041

$\sigma$  = standard deviation.

\* Gm. per kilo.

† Milliequivalents per kilo of water.

‡ Based upon twenty-three observations.

§ Gm. per 100 cc.

|| Cc. per 100 cc.

thirty-nine observations to be  $155.3 \pm 10.2$  and  $142.6 \pm 12.2$  milliequivalents per kilo of water, respectively. The latter values are in close agreement with the mean values for serum and cells, namely  $155.9 \pm 2.2$  and  $145.0 \pm 4.3$  milliequivalents per kilo of water, respectively, found in the present series. The value  $3.5 \pm 0.5$  milliequivalents per kilo of water for serum potassium given in Table I is somewhat lower than that found by

Hastings and Eichelberger (16), McIntyre (18), and others. The explanation for this difference is not entirely clear; however, it might be pointed out that the animals employed in the present study were receiving a low potassium diet. The cell potassium,  $8.9 \pm 1.1$  milliequivalents per kilo of water, is somewhat lower than the average value of 10.8 milliequivalents per kilo of water reported by McIntyre (18) for ten normal dogs.

Dill, Edwards, Florkin, and Campbell (15) compared the distribution of chloride and of bicarbonate between serum and cells in oxygenated blood, pH, 7.45, and found the chloride distribution ratio ( $r_{Cl}$ ) to be 0.65 and the bicarbonate distribution ratio ( $r_{HCO_3}$ ) to be 0.765. By comparing these values with those obtained on normal human subjects, the latter authors concluded that there is no significant difference between the two species either in the absolute magnitude of  $r$  or in the relation of  $r_{Cl}$  to  $r_{HCO_3}$ . From Table I it will be seen that the mean value for  $r_{Cl}$  was found to be  $0.682 \pm 0.016$  and that for  $r_{HCO_3}$  was found to be  $0.770 \pm 0.041$  at a mean pH, of 7.42. In this connection it might be pointed out that Hastings, Sendroy, McIntosh, and Van Slyke (14) reported the following mean values for  $r_{Cl}$  and  $r_{HCO_3}$  (mean pH, 7.38) for nine normal human subjects; namely, 0.689 and 0.786, respectively. In view of the similarity of the values above, the lines constructed in Fig. 1 to show the change of  $r_{Cl}$  and  $r_{HCO_3}$  with pH, in oxygenated blood have been taken from Dill, Edwards, and Consolazio (19) and represent the changes for human blood.

*Electrolyte and Water Content of Serum and Cells in Adrenal Insufficiency.* Serum—A summary of the data obtained from the analysis of serum and cells of eleven dogs in adrenal insufficiency is presented in Table II. By comparing the mean values given in Table II with those shown in Table I, it will be observed that there was a marked fall of the serum water and an increase of total protein. There was a decrease of the mean pH, of 0.21 and this was accompanied by deficits of chloride, bicarbonate, and sodium of 9.8, 10.6, and 16.0 milliequivalents per kilo of water, respectively. At the same time the mean serum potassium had risen by 5.6 milliequivalents per kilo of water. It will be noted that the sum of the mean deficits of chloride and bicarbonate exceeded that of sodium. An increase in the excess of determined

base over determined acid was encountered in nine of the eleven experiments. However, the undetermined acid value was markedly increased only in the terminal stages. It appears, therefore, that the acidosis, which may become extreme in terminal adrenal insufficiency (minimum values for  $pH_s$  of 7.0 and for bicarbonate of 7 milliequivalents were encountered), is the result of a relatively greater loss of sodium over chloride, an extension

TABLE II

*Electrolyte and Water Content of Serum and Cells of Eleven Dogs in Adrenal Insufficiency*

	Serum				Erythrocytes			
	Mean	Maximum	Minimum	$\sigma$	Mean	Maximum	Minimum	$\sigma$
Water*.....	899.0	914	883	9.3	664.4	676	653	7.0
$pH_s$ .....	7.21	7.3	7.0	0.12				
$BCl\uparrow$ .....	107.4	117	94	6.6	79.6	85	75	2.4
$BHCO_3\uparrow$ .....	12.5	18	7	3.3	10.5	13	6	3.1
$BPr\uparrow$ .....	19.7	24	16	2.1	44	56	28	10
Determined acid $\uparrow$ .....	139.6	151	127	7.1	134.1	147	116	10
$Na\uparrow$ .....	139.9	147	132	4.7	128.7 $\uparrow$	140	118	6.0
$K\uparrow$ .....	9.1	19	4	3.8	9.0 $\uparrow$	11	6	1.6
Determined base $\uparrow$ .....	149.0	164	138	5.7	137.7 $\uparrow$	150	126	6.9
Base minus acid $\uparrow$ .....	9.4	34	0	10.3	5.6 $\uparrow$	26	-12	10.4
Protein $\S$ .....	8.1	9	7	0.6				
Cell volume $\parallel$ .....					58.3	70	51	6.1

\* Gm. per kilo.

$\uparrow$  Milliequivalents per kilo of water.

$\uparrow$  Nine observations.

$\S$  Gm. per 100 cc.

$\parallel$  Cc. per 100 cc.

of base bound by protein, and an increase of the undetermined acid value.

The directions of the changes of the various serum constituents encountered in the present experiments agree with those reported by others (2, 4, 20-25) who have allowed dogs to go into insufficiency by discontinuing cortical extract administration.

*Cells*—The following changes of the mean cell values were found to accompany the serum water and electrolyte disturbances recorded above; namely, a gain of water (water was found in-

creased in nine instances, decreased in two, and unchanged in one), an increase of cell volume, little or no change of the chloride and potassium concentrations, and deficits of bicarbonate and sodium. The fall of the determined cell anion content was greater than that of determined base.

Although the anion and cation changes of the cells will be discussed later along with those encountered in other experiments, it should be pointed out that Nilson (6) observed a high level of potassium in the cells in acute adrenal insufficiency in dogs, caused by a high intake of potassium. This increase was noted just before crisis and the value fell rapidly following treatment. Some correlation between the changes of serum and cell sodium was reported and it appeared that the cell chloride level was maintained. Hegnauer and Robinson (26) studied the composition of the cells of cats in adrenal insufficiency and found a decrease in the concentration of cell sodium and an increase of cell potassium. In the present experiments the cell potassium did not change greatly. When the cell potassium concentration was corrected to a solid content equal to that of the control cells, a small absolute gain was observed.

*Electrolyte and Water Changes of Blood Following Cortical Extract Administration*—In the interest of observing the effect of the administration of cortical extract alone upon the electrolyte and water equilibria of the blood in adrenal insufficiency, arterial blood was obtained from four dogs in insufficiency and then again after treatment. Since a relatively large quantity of blood had to be obtained for the various analyses, it was found necessary to replace the blood withdrawn from the dogs in adrenal insufficiency by an equal quantity of normal dog blood. Immediately following the transfusion, 25 cc. of cortical extract were given intravenously.<sup>2</sup> At intervals over the next 40 hours additional 5 cc. quantities were injected subcutaneously, so that the animals received a total of between 50 and 60 cc. of extract. Neither food nor water was allowed and the final blood was obtained in each case 48 hours after the experiment had begun. At that time all the animals showed marked improvement and were quite active.

*Serum*—The results of the analysis of the serum and cells from

<sup>2</sup> Upjohn adrenal cortex extract.

TABLE III

Changes of Water and Electrolytes in Serum and Erythrocytes of Dogs in Adrenal Insufficiency Treated with Cortical Extract

	In insufficiency, before treatment				48 hrs. after treatment			
	Dog T	Dog U	Dog V	Average	Dog T	Dog U	Dog V	Average

Serum								
Water, gm. per kilo.....	901.0	889.5	896.0	898.0	896.1	905.3	887.5	895.5
pH,.....	7.32	7.16	7.28	7.06	7.21	7.24	7.25	7.37
BCl, m.eq. per kilo water.....	103.7	111.6	112.0	110.8	109.5	109.0	115.2	118.2
BHCO <sub>3</sub> , " " ".....	9.7	11.5	10.9	9.7	10.5	6.8	15.7	10.5
BPr, " " ".....	19.5	19.2	20.3	17.0	19.0	18.5	21.2	22.0
Determined acid, m.eq. per kilo water...	132.9	142.3	143.2	137.5	139.0	134.3	152.1	150.7
Na, m.eq. per kilo water.....	132.2	141.7	135.9	131.5	135.3	137.2	147.7	143.2
K, " " ".....	9.5	7.1	6.9	6.6	7.5	8.2	4.5	6.9
Determined base, m.eq. per kilo water...	141.7	148.8	142.8	138.1	142.8	145.4	152.2	150.1
Base minus acid, " " ".....	8.8	6.5	-0.4	0.6	3.8	11.1	0.1	-0.6
Protein, gm. per 100 cc.....	7.7	8.1	8.1	7.6	7.9	7.6	8.5	8.4

Cells								
Cell volume, cc. per 100 cc.....	67.5	51.0	56.7	53.5	57.2	64.4	50.2	57.3
Water, gm. per kilo.....	659.0	657.3	663.0	673.0	663.1	655.3	658.0	653.5
BCl, m.eq. per kilo water.....	75.1	82.0	80.6	81.6	79.8	74.4	84.6	85.4
BHCO <sub>3</sub> , " " ".....	8.2	9.0	8.7	7.7	8.4	5.1	12.1	9.3
BPr, " " ".....	55	40	50	29	44	47	48	59
Determined acid, m.eq. per kilo water...	138.3	131.0	139.3	118.3	131.7	126.5	144.7	153.7
Na, m.eq. per kilo water.....	118.0	127.7	125.4	121.0	123.0	121.7	133.8	137.1
K, " " ".....	8.4	9.9	8.1	10.8	9.3	10.5	10.1	6.2
Determined base, m.eq. per kilo water...	126.4	137.6	133.5	131.8	132.3	132.2	143.9	143.3
Base minus acid, " " ".....	-11.9	6.6	-5.8	13.5	0.4	5.7	-0.8	-10.4
Blood oxygen capacity, mm.....	12.3	9.4	10.5	9.5	10.4	12.4	9.3	10.4

Water, gm. per kilo.....	895.3	895.5	895.3	895.5
pH,.....	7.21	7.37	7.21	7.37
BCl, m.eq. per kilo water.....	114.4	118.2	114.4	118.2
BHCO <sub>3</sub> , " " ".....	12.7	10.5	12.7	10.5
BPr, " " ".....	19.9	22.0	19.9	22.0
Determined acid, m.eq. per kilo water...	147.0	150.7	147.0	150.7
Na, m.eq. per kilo water.....	140.9	143.2	140.9	143.2
K, " " ".....	5.6	6.9	5.6	6.9
Determined base, m.eq. per kilo water...	146.5	150.1	146.5	150.1
Base minus acid, " " ".....	-0.5	-0.6	-0.5	-0.6
Protein, gm. per 100 cc.....	8.1	8.4	8.1	8.4

Water, gm. per kilo.....	56.4	56.4	56.4	56.4
pH,.....	658.7	658.7	658.7	658.7
BCl, m.eq. per kilo water.....	82.0	82.0	82.0	82.0
BHCO <sub>3</sub> , " " ".....	9.3	9.3	9.3	9.3
BPr, " " ".....	49	49	49	49
Determined acid, m.eq. per kilo water...	140.3	140.3	140.3	140.3
Na, m.eq. per kilo water.....	131.1	131.1	131.1	131.1
K, " " ".....	9.0	9.0	9.0	9.0
Determined base, m.eq. per kilo water...	140.1	140.1	140.1	140.1
Base minus acid, " " ".....	-0.2	-0.2	-0.2	-0.2
Blood oxygen capacity, mm.....	10.4	10.4	10.4	10.4

these four dogs are recorded in Table III. It will be observed that associated with the striking clinical improvement of these animals there was little if any change of the serum water, serum total protein, and blood oxygen capacity. On the other hand there was an elevation of pH, in three of the four dogs and the serum sodium and chloride concentrations increased significantly in each experiment. The average gain of the serum sodium was 7.0 milliequivalents per kilo of water, while that of chloride was 4.7 milliequivalents. The serum bicarbonate was variable, increasing somewhat in two instances, falling in one, and remaining essentially unchanged in one. The average serum potassium level fell by 1.2 milliequivalents per kilo of water, a maximum decrease of 2.6 milliequivalents being observed in Dog U and no change in Dog V.

*Cell Changes*—The average cell volume showed an insignificant decrease of 0.8 cc. per 100 cc., the greatest change being 3.1 cc. per 100 cc. in Dog T. The average cell water decreased by 4.4 gm. per kilo, the value having decreased in three of the four dogs. In general, the changes of the cell bicarbonate, chloride, and sodium were in the same direction as those encountered in the serum. The average level of the cell sodium became increased by 8.1 milliequivalents per kilo of water, while that of the chloride became increased by 2.2 milliequivalents. The cell potassium was variable, tending to fall in two instances, to increase in the third, and to remain essentially unchanged in the fourth.

The results of the above experiments are of interest in the light of those obtained by other workers in similar studies. It has been shown by Harrop, Nicholson, and Strauss (22) and others that the serum electrolyte and water disturbances which are encountered in adrenal insufficiency are promptly returned to normal following the reinjection of extract if the animals are allowed free access to food and water. However, in other experiments designed to limit the intake of either water or food (or both) somewhat variable results have been obtained. Thus, Stahl, Atchley, and Loeb (23) observed the effect of administering a single large dose of cortical extract to a dog in adrenal insufficiency. The animal received neither food nor water during the experimental period after the injection. Despite a striking improvement in the general condition of the animal within 15 to 20 minutes, no



consistent changes in the serum sodium, potassium, or water content were observed in 6 hours following the injection. Harrop (27) also observed the effect of cortical extract alone on the blood changes of the dog in adrenal insufficiency. In the course of 24 hours during which food as well as fluid was withheld, but 50 cc. of extract in divided doses were injected subcutaneously, a prompt clinical improvement and a marked diuresis were observed. Among other changes, Harrop found that there was dilution of blood, a marked fall of the plasma potassium, and a gain of plasma sodium amounting to approximately 5 milliequivalents per liter. From measurements of the interstitial fluid volume by means of sodium thiocyanate it was concluded that the principal movement of this fluid is into the cells following the withdrawal of extract and from the cells to the interstitial spaces when the hormone is reinjected. In their studies Swingle, Parkins, Taylor, and Hays (28, 29) withheld food but allowed water during the experimental period following extract administration. Dogs in adrenal insufficiency with low levels of serum chloride and sodium (28) showed rapid recovery following extract administration. Following an experimental period of several days there was evidence of blood dilution; however, the levels of serum sodium and chloride remained unchanged, or tended to fall slightly. In later experiments (29) upon dogs in adrenal insufficiency with higher levels of serum sodium and chloride, a striking dilution of the blood and lowered levels of serum sodium, potassium, and chloride were observed 12 hours after extract administration. 24 hours later the blood had diluted and returned to approximately the normal condition, the serum potassium had also returned to normal, and the serum sodium and chloride levels had definitely increased. These authors concluded that the injections of cortical extracts mobilize the accumulations of the intracellular water and electrolytes such as sodium, chloride, and probably potassium and shift them from intracellular to extracellular compartments and the blood stream, thereby bringing about dilution and relief of symptoms.

A feature which stands out in the present experiments is that although there was marked improvement in the animals' condition there was no evidence of blood dilution. Equally interesting are the changes of the serum sodium and chloride. The concen-

tration of both increased in the face of a constant water content, making it appear as though sodium and chloride had merely been added. In seeking the source of the sodium and chloride it should be pointed out that the extract which was employed in these experiments contained 0.9 per cent NaCl. Consequently the animals received approximately 8 to 9 milliequivalents of salt in this manner. Further, since the dogs in adrenal insufficiency received normal blood in an amount equal to that withdrawn for analysis, some sodium chloride was received from this source. However, attention should be called to the experiments to be described in Paper II (7) in which the electrolyte and water contents of the skeletal muscles from these dogs and from dogs in adrenal insufficiency are compared. Evidence is presented that there is a greater contraction of the extracellular phase of the skeletal muscle of adrenal-insufficient dogs receiving extract than of those untreated. Further, during the recovery period the excretion of urine amounted to from 200 to 500 cc. Unfortunately no analyses were made of the latter specimens. However, with the evidence presented one might infer that interstitial fluid had entered the blood, followed by the excretion of water (and some electrolytes), with the partial retention of sodium and chloride. It is of some interest that the average gain of serum sodium was balanced by the average gains of chloride, bicarbonate, and base bound by protein.

The results of these experiments tend to minimize the importance of a correction of the water and electrolyte equilibria of the blood as a factor in the part played by the cortical extract in restoring the animal to normal. That is, such a correction follows secondarily to some other action or actions.

*Electrolyte and Water Changes Following Intraperitoneal Injection*—In order to induce electrolyte and water changes of the blood similar to those encountered in adrenal insufficiency three dogs were given an intraperitoneal injection of 5 per cent glucose and two dogs were given a similar injection of 5 per cent glucose containing 3.8 gm. of KCl per liter. Both solutions were injected in amounts of 100 cc. per kilo of body weight and the blood was withdrawn at from 4 to 4½ hours after the injection. The results of the analysis of serum and of the cell volume and cell water are presented in Table IV.

As has already been demonstrated by others, the direction of

TABLE IV

*Changes of Water and Electrolytes in Serum and Erythrocytes of Dogs Following Injection of 5 Per Cent Glucose and 5 Per Cent Glucose Containing Potassium Chloride*

Dogs K, M, and Q received intraperitoneal injection of 5 per cent glucose solution; Dogs Y and Z, intraperitoneal injection of 5 per cent glucose containing 3.8 gm. of KCl per liter.

	Controls					Experimental				
	Dog K	Dog M	Dog Q	Dog Y	Dog Z	Dog K	Dog M	Dog Q	Dog Y	Dog Z
Serum										
Water, gm. per kilo .....	925.5	914.5	918.5	917.5	913.0	885.3	890.5	881.0	871.5	888.5
pH.....	7.42	7.50	7.46	7.41	7.33	7.27	7.41	7.35	7.24	7.34
BCl, m.eq. per kilo water.....	114.5	118.1	115.9	116.2	120.9	100.2	102.1	100.1	112.2	112.8
BHCO <sub>3</sub> , " " .....	23.5	24.1	26.9	20.4	18.3		18.7	16.3	10.9	13.6
BPr, " " " .....	14.9	17.3	16.7	15.9	16.2	23.3	23.6	24.0	22.7	19.2
Determined acid, m.eq. per kilo water.....	152.9	159.5	159.5	152.5	155.4		144.4	140.4	145.8	145.6
Na, m.eq. per kilo water.....	152.8	156.7	156.2	152.2	154.1	140.9	140.9	141.7	153.5	146.7
K, " " " .....	3.0	4.1	3.8	3.9	3.3	3.2	3.3	3.2	4.8	5.7
Determined base, m.eq. per kilo water....	155.8	160.8	160.0	156.1	157.4	144.1	144.2	144.9	158.3	152.4
Base minus acid, " " " .....	2.9	1.3	0.5	3.6	2.0		-0.2	4.5	12.5	6.8
Cells										
Water, gm. per kilo .....	647.8	648.7	652.8	647.5	656.0	669.7	671.5	667.3	656.5	669.5
Cell volume, cc. per 100 cc.....	49.1	47.3	48.5	52.4	51.5	66.5	59.5	65.1	65.6	54.5

changes (with the exception of serum potassium) following a single intraperitoneal injection of 5 per cent glucose is similar to that encountered in adrenal insufficiency. It is worthy of note in contrast, however, that the fall of serum chloride is essentially equal to that of sodium, and the bicarbonate content is not decreased to the same extent. Moreover, with a fall of serum base the resulting changes of cell volume and cell water appear to be somewhat greater.

*Osmotic Adjustment between Serum and Cells*—The present experiments afford an opportunity to examine the manner in which osmotic equilibrium is brought about between serum and cells of dogs following rather marked distortions of the chemical patterns. Although this subject will be treated more critically in a later publication in connection with other data, it seems desirable to point out several interesting changes at this time.

The results of the analysis of the serum and cell base and of the cell water are presented in Table V. From the data obtained on the blood of dogs in adrenal insufficiency, it will be observed that while the cells generally gained water this gain was small in view of the extent of the serum sodium deficits. In this connection it will be noted that the fall of the serum sodium was accompanied by a decrease of the cell sodium concentration. Thus, by comparing the values presented in Tables I and II, it will be observed that the mean concentrations of serum and cell sodium fell by 16.0 and 16.3 milliequivalents per kilo of water, respectively. Since the serum potassium became elevated whereas the cell potassium concentration remained essentially unchanged, the average deficit of determined base in cells was greater than that in serum; *i.e.*, the serum base fell 10.4 milliequivalents, whereas the cell base fell 16.1 milliequivalents per kilo of water. In order to approximate the actual change of the cell base content, a constancy of the cell solids was assumed and the experimental cell values were corrected to a solid content equal to that of the control cells. From the values for " $B_2 - B_1$ " of Table V it will be seen that the cell base content was decreased in every instance, the maximum deficit being 26.8 milliequivalents per kilo of water in Dog T.

Another factor to be considered is the anion exchange between the serum and cells which accompanies the fall of pH. As

indicated above, the concentration of base in the cells fell to a greater extent than it did in the serum. When the sums of the mean deficits of base, chloride, and bicarbonate of the two phases are compared (Tables I and II), it will be found that the sum in serum had decreased by 30.8 milliequivalents per kilo of water, while that in the cells had decreased by 24.5 milliequivalents.

TABLE V

*Changes of Water and Base in Serum and Erythrocytes*

The first nine experiments represent a comparison of control (subscript 1) and blood of dogs in adrenal insufficiency (subscript 2). The last four represent comparisons of blood obtained before and following cortical extract administration.

The results are expressed in milliequivalents per kilo of water, except for  $(\text{H}_2\text{O})_1$  and  $(\text{H}_2\text{O})_2$  for which the readings are in gm. per kilo.

Dog	Serum				Cells							
	Na <sub>1</sub>	B <sub>1</sub>	Na <sub>2</sub>	B <sub>2</sub>	Na <sub>1</sub>	B <sub>1</sub>	Na <sub>2</sub>	B <sub>2</sub>	B <sub>2</sub> *	B <sub>2</sub> *-B <sub>1</sub>	(H <sub>2</sub> O) <sub>1</sub>	(H <sub>2</sub> O) <sub>2</sub>
A	158.9	163.6	142.8	152.0	152.0	159.1	128.5	134.1	146.4	-12.7	650.7	670.5
D	158.1	160.5	143.5	147.4	146.0	156.6	136.3	144.8	152.2	-4.4	651.5	662.8
N	153.5	157.5	140.5	149.3	143.1	153.1	132.0	142.0	142.5	-10.6	661.0	661.8
R	154.8	158.3	146.7	154.5	146.2	155.6	140.4	150.4	147.1	-8.5	657.5	652.5
S	155.8	159.2	138.5	149.6	150.4	158.6	128.8	138.9	143.9	-14.7	652.5	660.5
T	152.0	156.2	132.2	141.7	144.4	152.4	118.0	126.4	125.6	-26.8	660.5	659.0
U	157.8	162.0	141.7	148.8	149.4	158.6	127.7	137.6	142.1	-16.5	650.0	657.3
V	154.2	158.1	135.9	142.8	149.3	156.9	125.4	133.5	142.5	-14.4	648.3	663.0
W	153.0	155.6	131.5	138.1	144.3	154.3	121.0	131.8	139.1	-15.2	661.0	673.0
T	132.2	141.7	137.2	145.4	118.0	126.4	121.7	132.2	130.0	3.6	659.0	655.3
U	141.7	148.8	147.7	152.2	127.7	137.6	133.8	143.9	144.3	6.7	657.3	658.0
V	135.9	142.8	143.2	150.1	125.4	133.5	137.1	143.3	137.4	3.9	663.0	653.5
W	131.5	138.1	140.9	146.5	121.0	131.8	131.8	141.0	137.7	5.9	673.0	667.8

\* Values corrected to a cell solid content equal to that of the control.

This relatively greater loss of anions from serum can be accounted for by a retention of anions in the cells to combine with base set free by the cell proteins as a result of the fall in pH.

These experiments indicate that the chemical pattern of the blood cells of dogs may undergo wide changes in an attempt to come into equilibrium with the serum. Following the serum electrolyte and water deficits encountered in adrenal insufficiency, the establishment of equilibrium is apparently associated with a

loss of base (sodium) from the cells and with anion and water exchange.

It will be appreciated that in this study the control and experimental bloods were obtained a number of days apart. Therefore, the following questions arise. Are the apparent losses of cell sodium to be interpreted as evidence supporting the view that the corpuscular membrane is permeable to this ion? Or have new cells of a different cation content entered the circulation in the interval between sample collections? The same questions were considered by Robinson and Hegnauer (30) who also observed a loss of sodium from the red blood cells of cats in adrenal insufficiency. By using intraperitoneal injections, the latter authors found the cell changes to be similar to those encountered in adrenal insufficiency. Consequently it was concluded that, when the plasma electrolyte balance is sufficiently altered, the red blood cell membrane may become somewhat permeable to cations. Yannet, Darrow, and Cary (17) found that a passage of sodium across the red cell membrane occurred in dogs following the intraperitoneal injection of a 5 per cent solution of glucose and following a similar injection of a 1.8 per cent solution of sodium chloride.

The four experiments of the present study dealing with the effect of cortical extract alone on the water and electrolyte changes are of interest in this connection. It was pointed out previously that associated with the average gain of serum sodium of 7.0 milliequivalents per kilo of water (Table III) the average cell sodium concentration increased by 8.1 milliequivalents. Further, the average gain of determined serum base was 5.8 milliequivalents, while that of the cells was 7.8 milliequivalents per kilo of water. From the values given under " $B_2 - B_1$ " in Table V, it will be observed that in each experiment there was evidence of an absolute gain of the cell base. This occurred in the face of a slight fall of the cell water. On the other hand, when the average gains of the sums of base, chloride, and bicarbonate of the two phases are compared (Table III), it will be seen that the sum increased in serum by 11.4 milliequivalents per kilo of water, while that in the cells increased by 10.9 milliequivalents. Therefore, in these experiments as in the case of the blood in adrenal insufficiency, the establishment of equilibrium between serum and

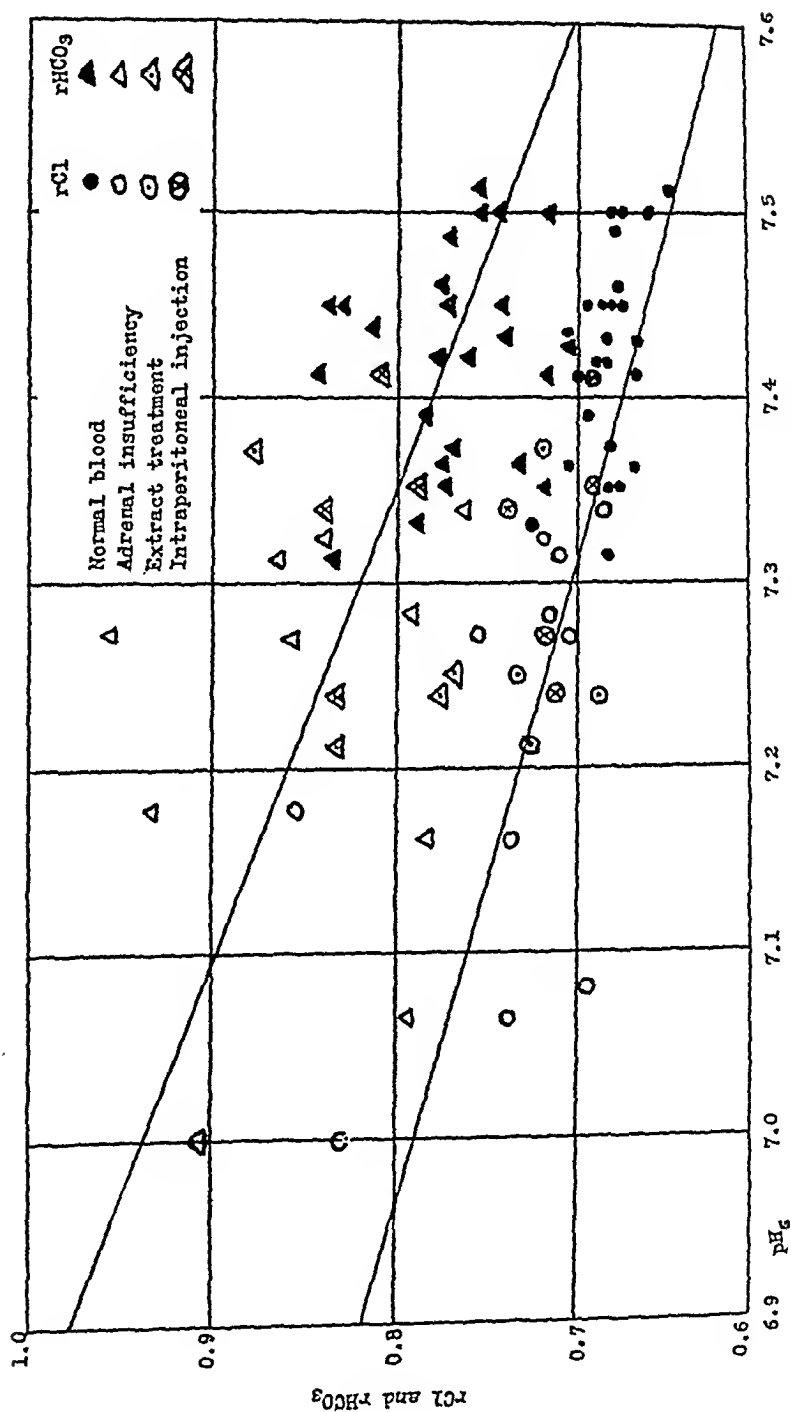


Fig. 1. Change in the chloride and bicarbonate distribution ratios with pH.

cells was apparently associated with a passage of sodium across the membrane and with anion and water exchange.

*Distribution of Chloride and Bicarbonate between Serum and Cells*—Van Slyke, Wu, and McLean (5) considered the factors responsible for the unequal distribution of diffusible ions between serum and cells and showed that the distribution in oxygenated blood is related to the non-diffusible ions by the equation

$$\frac{[\text{H}^+]_c}{[\text{H}^+]_s} = \frac{[\text{Cl}^-]_c}{[\text{Cl}^-]_s} = \frac{[\text{HCO}_3^-]_c}{[\text{HCO}_3^-]_s} = 1 - \frac{[\text{BPr}]_c + [\text{Hb}]_c - [\text{BPr}]_s}{2([\text{B}]_s - [\text{BPr}]_s)} \quad (1)$$

Subsequent work from various laboratories has shown that, although the determined molal ratios  $[\text{Cl}]_c/[\text{Cl}]_s$  and  $[\text{HCO}_3]_c/[\text{HCO}_3]_s$  are not equal, they vary with the  $\text{pH}_s$ , as predicted by

TABLE VI

*Determined and Calculated Distribution Ratios (Average Values)*

$\text{pH}_s$ range	$\text{pH}_s$	Observed		Calculated $r^*$
		$r_{\text{Cl}}$	$r_{\text{HCO}_3}$	
7.0 – 7.20(5)†	7.10	0.770	0.853‡	0.843
7.20 – 7.40(22)	7.31	0.703	0.807	0.781
Above 7.40(17)	7.45	0.674	0.771	0.721

\* Calculated by substituting average values in Equation 1.

† The figures in the parentheses indicate the number of observations taken to establish the average.

‡ Only four observations.

the equation. Since the theory assumes an impermeability of the cell membrane to cations, it was of interest to observe the changes of  $r_{\text{Cl}}$  and  $r_{\text{HCO}_3}$  with  $\text{pH}_s$  encountered in the present experiments, where some evidence of sodium transfer was obtained.

In Fig. 1 the chloride and bicarbonate ratios plotted against  $\text{pH}_s$  are given for normal dogs, dogs in adrenal insufficiency, and following cortical extract administration, and following the intraperitoneal injections of glucose or glucose containing potassium chloride. As stated previously, the lines have been constructed from the data of Dill, Edwards, and Consolazio (19) and represent the changes for oxygenated human blood. It will be observed that while some scattering of points is evident, par-



ticularly in the  $r\text{HCO}_3$  values, they tend to fall reasonably well along the lines. Somewhat greater variation in the  $r\text{HCO}_3$  values might be anticipated, since the cell bicarbonate content was obtained only by indirect calculation. In order to determine in another way whether the changes are in a direction predicted by Equation 1, the values for  $r\text{Cl}$  and  $r\text{HCO}_3$  in a given pH range were averaged and compared with the average calculated value. From the data presented in Table VI, it will be observed that the values for  $r$  calculated by Equation 1 fall in between the average determined values. Further, the changes of calculated  $r$  with pH, approximately parallel the lines constructed in Fig. 1. The average determined  $r\text{Cl}$  and  $r\text{HCO}_3$  values, with the exception of  $r\text{HCO}_3$  at the average pH, of 7.10, agree rather closely with the corresponding values indicated by the lines. On the whole it appears that, regardless of the way in which the blood electrolyte changes were induced, the distribution of anions between serum and cells was influenced chiefly by the factors indicated by Equation 1.<sup>3</sup>

#### SUMMARY AND CONCLUSIONS

The results of the analysis of the water and electrolyte content of serum and cells of twenty-four normal dogs, of eleven dogs in adrenal insufficiency, of four dogs in adrenal insufficiency following cortical extract administration, and of five dogs receiving intraperitoneal injections of either glucose or glucose containing potassium chloride have been presented. The various findings have been discussed briefly and may be summarized as follows:

1. *Blood Changes in Adrenal Insufficiency*—The results of the analysis of the serum of dogs in adrenal insufficiency revealed a fall of the water content and a gain of total protein. There were also a fall of pH, decreases of chloride, bicarbonate, and sodium, and an increase of potassium. The red blood cells in adrenal

<sup>3</sup> Since the completion of this paper, a relevant publication by Rapoport and Guest (31) has appeared. It is shown that the distribution ratios, predicted by the formulae of Van Slyke, Wu, and McLean, for  $\text{H}^+$ ,  $\text{Cl}^-$ , and  $\text{HCO}_3^-$  (corrected for carbamate  $\text{CO}_2$  in the cells) between the serum and cells of dogs, in normal condition and following pyloric obstruction, are brought nearer the observed values if the equivalencies of non-diffusible cell constituents of the original equation are taken as the sum of the equivalents of hemoglobin, diphosphoglycerate, and "other organic P."

insufficiency usually gained water, the bicarbonate and sodium concentrations were reduced, and the chloride and potassium concentrations were only slightly changed.

From the serum changes it was concluded that the acidosis encountered was the result of a relatively greater loss of sodium over chloride, an extension of base bound by protein, and an increase of the undetermined acid value.

*2. Blood Changes Following Cortical Extract Administration—*

There was a marked clinical improvement of the four animals in adrenal insufficiency upon treatment with cortical extract alone. No evidence of blood dilution was obtained, since there was little if any change of the serum water, serum total protein, or blood oxygen capacity. Despite a relative constancy of the serum water, there were appreciable gains of the serum sodium and chloride concentrations, some increase of the average pH, and of the average bicarbonate content. The serum potassium tended to fall. There was some tendency for the cells to lose water and in general the variations of the cell bicarbonate, chloride, and sodium were in the same direction as those encountered in the serum. The changes of cell potassium were variable. Evidence was presented that the increases in the concentrations of serum sodium and chloride may be interpreted as resulting from an entrance of interstitial fluid into the circulation followed by an excretion of water (and some electrolytes) with the (partial) retention of sodium and chloride.

It was concluded that these experiments tend to minimize the importance of a correction of the water and electrolyte equilibria of the blood as a factor in the part played by the cortical extract in restoring the animal to normal. Such a correction follows secondarily some other action or actions.

*3. Anion and Cation Equilibria between Serum and Cells—*The chemical pattern of the red cells of dogs may undergo rather wide changes in coming into equilibrium with the serum. Evidence was presented that the establishment of osmotic equilibrium between the serum and cells of dogs in adrenal insufficiency is associated with a loss of base (sodium) from the cells and with anion and water exchange. Following cortical extract administration, the equilibrium was associated with a gain of cell sodium and with anion and water exchange.

Despite the evidence of a change of the cell base content, the distribution ratios  $r_{\text{Cl}}$  and  $r_{\text{HCO}_3}$  varied with pH, reasonably well, in accord with the theory that the diffusible ions are influenced by the non-diffusible ions according to the Gibbs-Donnan law.

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## ELECTROLYTE AND WATER EQUILIBRIA IN THE DOG

### II. ELECTROLYTE AND WATER EXCHANGE BETWEEN SKELETAL MUSCLE AND BLOOD IN ADRENAL INSUFFICIENCY\*

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In recent years a number of workers have been interested in studying the water and electrolyte changes of the blood and tissues encountered in experimental adrenal insufficiency and in observing the effect of administering cortical extract upon such changes. Rather marked blood electrolyte disturbances are observed in adrenal insufficiency in dogs and the constant finding of a blood concentration indicates that a change of body water is a characteristic feature in such animals. Balance studies with adrenalectomized dogs (2) or with adrenalectomized dogs after injections of extract were stopped (3) showed that a moderate diuresis usually accompanies an increased excretion of sodium. Swingle, Pfiffner, Vars, and Parkins (4) concluded that while such loss of fluid by way of the urine plays a part it is not the sole factor in the dehydration of adrenal insufficiency. These authors suggested that there is a disturbance of the normal fluid exchange between the blood and the tissues. That is, there is an accumulation of fluid in the tissues and interstitial spaces. As a result of further study Swingle, Parkins, Taylor, and Hays (5, 6) have continued to stress the feature that in adrenal insufficiency there is an impairment of the mechanism regulating and controlling the internal fluid distribution of the body. It was concluded (5) that the disappearance of symptoms, when adequate amounts of cortical hormone are administered, is largely due to the effect

\* A preliminary report of a part of this work was presented before the American Society of Biological Chemists (1).

of the cortical hormone on the mobilization and shift of tissue fluids to the extracellular spaces and blood stream. The movement of the water from the cells in this case apparently occurs independently of the level of sodium in the serum. Darrow and Yannet (7) and Gilman (8) noted that the blood electrolyte and water changes and other phenomena observed in intact animals given injections of intraperitoneal glucose are similar in many respects to those encountered in adrenal insufficiency. Consequently it was suggested that the loss of sodium in insufficiency unaccompanied by a corresponding diminution of body water brings about a transfer of water from the extracellular spaces to the tissue cells to maintain osmotic equilibrium. Harrop (9) found that an actual shrinkage of interstitial fluid volume occurs during insufficiency and that this volume is restored by reinjection of hormone. In agreement with the views of Darrow and Yannet and Gilman, Harrop concluded that the movement of extracellular fluid into the tissue cells as a result of a loss of extracellular electrolyte is the most important factor leading to blood concentration.

The recent studies of the electrolyte and water content of the skeletal muscle of cats (10, 11), rats (12), and dogs (11) in adrenal insufficiency have yielded results which support the view that the intracellular changes are associated with those observed in the extracellular fluids. An outstanding feature of the latter studies has been the finding of a gain of potassium in the muscle cells. In view of the evidence of increased toxicity of potassium in adrenalectomized animals and in patients with Addison's disease (13) this observation commands considerable interest.

The experiments included in this paper were carried out in conjunction with those reported in Paper I (14) and were undertaken for the following purposes: (1) to obtain further information regarding the extent of the electrolyte and water disturbances of the skeletal muscle of dogs in adrenal insufficiency and to approximate, by the procedure outlined by Hastings and Eichelberger (15), the changes of the volumes of the extra- and intracellular phases of muscle; (2) to compare the skeletal muscle changes observed in adrenal insufficiency with those encountered in dogs following intraperitoneal injections of glucose and glucose containing potassium chloride, and following a high potassium intake; and (3) to observe the effect of the administration of

cortical extract alone upon the muscle disturbances encountered in adrenal insufficiency.

### *Methods*

The care of the animals employed in the present studies and the treatment of the blood specimens were briefly described in Paper I (14). Skeletal muscle (lumbar portion, sacrospinalis) was obtained under sodium pentobarbital anesthesia immediately following the collection of the blood. It was quickly freed of adherent blood and immediately placed in a glass-stoppered bottle. In a specially constructed humidor, the muscle was trimmed to remove as much free fat and connective tissue as possible, finely minced with a pair of scissors, and placed in a weighed covered beaker. The sampling was carried out as rapidly as possible.

Weighed aliquot samples were used to determine the water content by drying to constant weight at 105–110°. The neutral fat was determined in the same way as described by Hastings and Eichelberger (15) on the residue remaining in the weighing bottles after the evaporation of water. The residue remaining after the fat extraction was then subjected to a Kjeldahl nitrogen estimation. Weighed aliquot samples were also employed to determine the collagen nitrogen by the method of Spencer, Morgulis, and Wilder (16) (the nitrogen, however, was determined by micro-Kjeldahl procedure) and the chloride by the method of Sunderman and Williams (17). The muscle sample remaining in the beaker was dried to constant weight at 105–110° and ashed in the same way as described by Cullen and Wilkins (18). Sodium was determined on an aqueous solution of the ash by the method of Butler and Tuthill (19) and potassium by the cobaltinitrite technique (20). All the determinations were carried out at least in duplicate.

Unless stated otherwise, the volumes of extra- and intracellular phases of muscle were calculated in the manner outlined by Hastings and Eichelberger (15) which is based on the assumption that all the chloride is extracellular and is present at a concentration equal to that of an ultrafiltrate of serum. In order to approximate the changes of the muscle phases the calculations were made relative to the control series and a constant solid content



of the intracellular phase assumed. The symbols used in the present report are the same as those employed by Hastings and Eichelberger (15).

# EXPERIMENTAL

*Electrolyte and Water Content of Normal Dog Muscle*—A summary of the data obtained from the analysis of the skeletal muscle of twenty-four normal dogs is given in Table I. Since Hastings and Eichelberger (15) have reported similar data from the analysis

TABLE I

## *Electrolyte and Water Content of Normal Dog Muscle\**

The values are expressed in terms of 1000 gm. of fat-free muscle.

	Mean	Maximum	Minimum	Standard deviation
Chloride, <i>m.eq.</i> .....	19.5	26	15	2.7
Sodium, <i>m.eq.</i> .....	28.2	37	22	3.2
“ minus chloride, <i>m.eq.</i> .....	8.7	12	5	1.9
Potassium, <i>m.eq.</i> .....	83.5	95	69	5.5
Water, <i>gm.</i> .....	761.4	780	749	7.8
Extracellular phase, ( <i>F</i> ), <i>gm.</i> .....	159	218	119	22
Intracellular water, ( $H_2O$ ) <sub>c</sub> , <i>gm.</i> ...	604	639	549	18
“ “ { $H_2O$ } <sub>c</sub> , <i>gm.</i> †..	718	733	703	8
Collagen nitrogen, ‡ <i>gm.</i> .....	4.88	5.7	3.6	0.6
Total nitrogen, <i>gm.</i> .....	34.59	37.7	32.2	1.61

This table has been constructed on the basis of twenty-four observations.

\* Lumbar portion, sacrospinalis.

† Per 1000 gm. of muscle cells.

‡ Sixteen observations.

of the rectus abdominis muscle of normal dogs, it is of interest to make a comparison of results. In general there is a fair agreement between the two series of observations. However, the mean values obtained in the present study for chloride, sodium, and water are somewhat lower, while that for potassium is slightly higher than the corresponding mean values reported by the above workers. The calculation of the extracellular phase (*F*) yielded a mean value of 159 gm., with a standard deviation ( $\sigma$ ) of  $\pm 22$  gm., per kilo of muscle. Thus, as a result of the lower chloride content observed in the present series, the latter value is somewhat smaller

than the value ( $F$ ) = 173 gm.,  $\sigma \pm 22$ , found by Hastings and Eichelberger. In agreement with the latter authors the per cent of water in the intracellular phase is relatively constant. The value for  $\{H_2O\}_c$  of 718 gm.,  $\sigma \pm 8$ , per kilo of muscle cells compares favorably with their value of  $\{H_2O\}_c$  of 717 gm.,  $\sigma \pm 5$ .

At this point attention should be called to the recent studies by Manery, Danielson, and Hastings (21) which indicate that the extracellular phase of muscle may be more accurately described as consisting of extracellular fluid and connective tissue proteins. By assuming that the connective tissue of muscle corresponds to tendon and is diluted with serum ultrafiltrate in an amount determined by the muscle chloride in excess of that accounted for by the connective tissue, the above authors obtained the following results by calculation. It was found that of a kilo of rabbit muscle the connective tissue phase amounts to 156 gm., containing 78.1 per cent water, compared with the extracellular phase of 131 gm., containing 99 per cent water, calculated from the ultrafiltrate chloride concentration. In other words, the magnitude of the phase and its per cent of water are altered when the calculations are made on the basis of the above assumptions.

In a number of the present experiments an attempt was made to approximate the connective tissue content of dog muscle by determining the collagen nitrogen. From Table I it will be observed that a mean collagen nitrogen content of 4.88 gm. per kilo of muscle was obtained from sixteen observations. The analysis of tendon from normal dogs (22) yielded average chloride and water contents of 78.7 milliequivalents and 668.3 gm. per kilo, respectively (corrected for neutral fat). From these data and by making the assumptions that the connective tissue of muscle corresponds to tendon and that the partition of collagen N to total N is the same for dog tendon as for beef tendon (23), it is possible to approximate the connective tissue content and the portions of water and chloride it accounts for. The chloride of muscle in excess of that accounted for by the connective tissue is used to calculate the ultrafiltrate volume. By making such calculations the following results were obtained. The mean connective tissue phase (corresponding to the extracellular phase ( $F$ )) amounted to 187 gm.,  $\sigma \pm 21$ , per kilo of muscle, containing 84.6 per cent water. This increase in the magnitude of the extra-

cellular phase obviously causes a corresponding decrease of the magnitude of the intracellular phase. As a result the mean water content of the muscle cells amounted to 742 gm.,  $\sigma \pm 9$ , per kilo, compared with the mean value 718 gm.,  $\sigma \pm 8$ , given in Table I.

It will be observed, as was pointed out by Manery, Danielson, and Hastings (21), that such calculations yield values for the extracellular phase and for the per cent of water in the intracellular phase which are somewhat greater than those obtained from the ultrafiltrate chloride concentration alone. In the present study the collagen nitrogen was also determined on a number of experimental muscle samples. For the sake of comparison, in these instances the changes of the muscle phases were calculated in the usual manner (15) and also by taking into account the approximate amount of connective tissue. As will be pointed out later, the magnitudes of the phase changes differ somewhat; however, the directions are the same and lead to the same interpretations.

*Animals in Adrenal Insufficiency*—The results of the analysis of the muscles from seven dogs in adrenal insufficiency are presented in Table II. By comparing these values with those given in Table I, it will be seen that a loss of sodium and a gain of water are outstanding features. Associated with these changes there was a tendency for the chloride content to fall and the potassium to increase. In each experiment the loss of sodium was greater than that of chloride, resulting in a significant lowering of the value of sodium in excess of chloride. The gain of muscle water was the result of an increase of water in the intracellular phase. Thus, the average intracellular water,  $(H_2O)_i$ , was increased to 641 gm. per kilo of muscle. The magnitude of the extracellular phase,  $(F)$ , tended to fall.

In order to approximate the changes of the muscle phases the calculations were made relative to the control series and a constant solid content of the intracellular phase was assumed. Such calculations revealed that on the average an absolute increase of 67 gm. per kilo of control muscle,  $\Delta M$ , had occurred. This consisted of an average 80 gm. increase in the intracellular phase,  $\Delta C$ , and an average 13 gm. decrease in the extracellular phase,  $\Delta F$ . Thus, the absolute change in the bulk of a kilo of muscle may be attributed chiefly to a gain of intracellular water. The

changes in the extracellular phase were somewhat less significant. In all the animals, except Dog A, there was a tendency toward a decrease in the extracellular phase. However, this decrease was of real significance in only two instances; *i.e.*, Dogs D and S.

TABLE II

*Electrolyte and Water Content of Muscle from Dogs in Adrenal Insufficiency*

The values are expressed in terms of 1000 gm. of fat-free muscle.

	Dog A	Dog C	Dog D	Dog E	Dog N	Dog R	Dog S	Average
Chloride, <i>m.eq.</i> .....	16.0	13.3	12.5	15.0	16.5	16.2	16.1	15.1
Sodium, <i>m.eq.</i> .....	17.3	12.3	18.1	18.3	17.1	17.5	17.2	16.8
“ minus chlo- ride, <i>m.eq.</i> .....	1.3	-1.0	5.6	3.3	0.6	1.3	1.1	1.7
Potassium, <i>m.eq.</i> ....	73.3		86.3	83.3	95.2	91.9	95.7	87.6
Water, <i>gm.</i> .....	788.3	777.7	773.5	781.5	776.5	769.5	767.5	776.4
Extracellular phase, ( <i>F</i> ), <i>gm.</i> ...	155	136	107	142	148	139	132	137
Intracellular water, ( $H_2O$ ), <i>gm.</i> .....	635	643	668	641	630	632	637	641
Collagen nitrogen, <i>gm.</i> .....					7.26	5.78	4.97	6.00
$\Delta M^*$ .....	127	72	51	91	67	34	25	67
$\Delta C$ .....	112	86	98	96	69	50	49	80
$\Delta F$ .....	15	-14	-47	-5	-2	-16	-24	-13
$\Delta'M$ .....					153	65	31	83
$\Delta'C$ .....					131	73	54	86
$\Delta'F$ .....					22	-8	-23	-3

\* The changes of the muscle phases relative to the control series have been calculated on the assumption of a constant solid content of the intracellular phase. In calculation of the changes marked  $\Delta'$  the same assumption was made; however, in addition account was taken of the connective tissue content.

The collagen nitrogen was determined in three of these experiments, thus permitting an approximation of the changes of the muscle phases on the basis of the connective tissue contents. Such calculations lead to the same conclusion as was arrived at above; namely, that in the absolute increase in the bulk of the muscle the increase in the intracellular phase was the outstanding feature.

In the present experiments the muscle samples were obtained

in most instances following a period of several days during which the animals refused food. In order to observe the electrolyte and water changes of the muscle in normal animals following privation of food, three dogs were allowed water but no food for a period of 5 days (Table III). The results of the analysis of the muscles from two dogs (Dogs O and X) following a period of 20 days during

TABLE III

*Electrolyte and Water Content of Muscle from Dogs Following 5 Days Fasting and Following Ingestion of Potassium Acid Phosphate*

The values are expressed in terms of 1000 gm. of fat-free muscle.

	Dog F	Dog G	Dog I	Dog O	Dog X
Chloride, <i>m.eq.</i> .....	21.9	21.3	17.5	16.9	17.5
Sodium, <i>m.eq.</i> .....	29.3	26.8	23.8	23.9	24.6
“ minus chloride, <i>m.eq.</i> .....	7.4	5.5	6.3	7.0	7.1
Potassium, <i>m.eq.</i> .....	80.8	88.1	92.7	81.6	84.3
Water, <i>gm.</i> .....	773.0	765.0	762.5	759.3	756.0
Extracellular phase, ( <i>F</i> ), <i>gm.</i> .....	190	169	142	139	145
Intracellular water, ( $H_2O$ ) <sub>c</sub> , <i>gm.</i> .....	585	597	622	622	613
Collagen nitrogen, <i>gm.</i> ...				4.44	4.24
$\Delta M^*$ .....	52	16	3	-10	-23
$\Delta C$ .....	12	3	20	12	-5
$\Delta F$ .....	40	13	-17	-22	-18
$\Delta'M$ .....				-22	-41
$\Delta'C$ .....				5	-15
$\Delta'F$ .....				-27	-26

Dogs F, G, and I were allowed water but no food for a period of 5 days. Dogs O and X were studied following a period of 20 days during which increasing amounts of  $KH_2PO_4$  were included daily in the diet, beginning with 0.5 gm. and reaching a maximum of 25 gm. of the salt daily.

\* See the comments below Table II.

which increasing amounts of potassium acid phosphate were included daily in the diet are also included in Table III. It will be observed that no striking changes of the electrolyte and water content from normal were encountered in these experiments. Attention might be called to the slightly higher levels of potassium in two of the three dogs following the period of fasting and the

tendency toward slightly lowered levels of sodium and chloride following the period of the potassium salt ingestion. On the whole the calculated changes of the muscle phases were not great. The greatest change occurred with Dog F for which there was an absolute gain of 52 gm. in the bulk of the muscle, due chiefly to an increase in the extracellular phase. Following the administration of the potassium salt there was some tendency to an absolute decrease in the bulk of the muscle, due apparently to a decrease in the extracellular phase.

*Animals Following Intraperitoneal Injection*—In the interest of comparing the electrolyte and water changes of the muscle accompanying a rapid depletion of blood electrolytes with those encountered in adrenal insufficiency, several animals were subjected to intraperitoneal injection experiments. The results of the analysis of three dogs (Dogs K, M, and Q) following the intraperitoneal injection of 5 per cent glucose and two dogs (Dogs Y and Z) following a similar injection of 5 per cent glucose containing 3.8 gm. of KCl per liter of solution are presented in Table IV. In each instance 100 cc. of solution per kilo of body weight were injected and the samples were collected at from 4 to 4½ hours following the injection. By comparing the data presented in Table IV with those given in Table II, it will be observed that the directions of the changes following the intraperitoneal injection of glucose are similar in many respects to those encountered in adrenal insufficiency. There was a fall of the sodium content which was somewhat greater than that of the chloride, tending, therefore, to decrease the value for the excess of sodium over chloride. The potassium content remained within the normal range of variation. The calculations of the changes of the muscle phases relative to the control indicated an absolute gain in the bulk of the muscle. This gain was apparently the result of an increase in the intracellular phase and a relatively smaller decrease in the extracellular phase. It will be noticed that, while the direction of change is similar to that encountered in adrenal insufficiency, the decrease in the extracellular phase following the intraperitoneal injection of the glucose solution was more definite. The calculation of the changes of the muscle phases following the intraperitoneal injection of the solution containing glucose and potassium chloride indicated a decrease in the bulk

of the muscle. This was apparently the result of a decrease in the extracellular phase and a relatively smaller increase in the intracellular phase.

It is of interest to compare the skeletal muscle changes in adrenal insufficiency observed in the present study with those

TABLE IV

*Electrolyte and Water Content of Muscle from Dogs Following Intraperitoneal Injections*

The values are expressed in terms of 1000 gm. of fat-free muscle.

	Dog K	Dog M	Dog Q	Dog Y	Dog Z
Chloride, <i>m.eq.</i> .....	12.7	14.8	10.5	11.6	13.7
Sodium, <i>m.eq.</i> .....	15.7	20.7	13.1	17.4	20.5
“ minus chloride, <i>m.eq.</i> .....	3.0	5.9	2.6	5.8	6.8
Potassium, <i>m.eq.</i> .....	82.2	80.3	87.4	96.6	82.0
Water, <i>gm.</i> .....	770.3	769.5	765.8	753.8	762.8
Extracellular phase, ( <i>F</i> ), <i>gm.</i> .....	122	140	101	99	117
Intracellular water, ( <i>H<sub>2</sub>O</i> ) <sub>c</sub> , <i>gm.</i> .....	650	631	666	656	647
Collagen nitrogen, <i>gm.</i> ....	4.84	4.33	4.46	4.23	4.02
$\Delta M^*$ .....	37	34	16	-34	4
$\Delta C$ .....	70	49	73	30	46
$\Delta F$ .....	-33	-15	-57	-64	-42
$\Delta' M$ .....	41	24	7	-53	-17
$\Delta' C$ .....	82	51	76	20	35
$\Delta' F$ .....	-41	-27	-69	-73	-52

Dogs K, M, and Q were given intraperitoneal injections of 5 per cent glucose and Dogs Y and Z received similar injections of 5 per cent glucose containing 3.8 gm. of KCl per liter of solution in amounts of 100 cc. per kilo of body weight. The samples were obtained at from 4 to 4½ hours after the injection.

\* See the comments below Table II.

reported by others. Hegnauer and Robinson (10) experimenting with cats noted an increase of the potassium content of the muscles in insufficiency. The total water content of the muscle was found to increase or to remain constant, and when an increase was encountered it was intracellular. In the muscles of adrenalectomized cats, Darrow, Harrison, and Taffel (11) found a significant

increase in water and potassium and but little change in chloride, sodium, phosphorus, and protein, the various values being expressed in terms of 100 gm. of fat-free solids. It was observed further that both the intracellular and extracellular water increased by about 10 per cent; however, owing to the relatively greater volume of the intracellular water in skeletal muscle, the larger part of the increase in total water was intracellular. Despite this apparent dilution of the cell solutes, the concentration of potassium in the intracellular water was found to remain unchanged. The latter authors observed that the changes in the skeletal muscles of adrenalectomized dogs are essentially the same as those in adrenalectomized cats. That is, the increase of muscle water was found to be chiefly intracellular, and the concentrations of potassium in the intracellular water remained constant. In the skeletal muscle of rats in adrenal insufficiency Harrison and Darrow (12) found the concentrations of water and chloride to be unaltered, the sodium to be reduced, and the potassium to be strikingly elevated. It was concluded that the water content of the muscle cell is inversely related to the concentration of sodium in the extracellular water. The results of the later experiments by Darrow, Harrison, and Taffel (11) on cats and dogs in adrenal insufficiency were also taken to support the concept that a lowering of the osmolar concentration of the extracellular fluids accounts for the increase in the intracellular water in the muscles of such animals. That is, the transfer of water between the extra- and intracellular phases of skeletal muscle is the chief factor in the adjustment following a disturbance of osmolar concentrations. Harrop (9) found in adrenal insufficiency in the dog that there is a decrease of the total volume of extracellular fluid. Since in their experiments no decrease in the volume of the extracellular fluid of skeletal muscle (and liver) of animals in adrenal insufficiency was observed, Darrow, Harrison, and Taffel (11) pointed out that reservoirs of extracellular fluid other than those of muscle (and liver) must account for the decrease in the volume of extracellular fluids. The latter authors suggested that subcutaneous tissues give up considerable amounts of water as well as sodium and chloride.

In general, the results of the present experiments lend support to the concept that the changes of water in the skeletal muscles of



dogs in adrenal insufficiency are associated with the electrolyte and water changes of the extracellular fluids. In agreement with the observations of Darrow and his coworkers (11, 12), a fall of the concentration of sodium in the extracellular water is associated with a gain of intracellular water (decrease of solids). In contrast with the findings of the latter workers, however, some tendency toward a decrease in the extracellular phase of the skeletal muscle in adrenal insufficiency was observed in the present experiments. This was accompanied by a definite fall of the sodium concentration and a tendency toward a decrease of the chloride concentration. The changes of the potassium content of the muscles are presented in Table V. The values are expressed in terms of 1000 gm. of intracellular water. It will be observed that relative to the control value the concentration of potassium in the intracellular water in adrenal insufficiency was lowered in three experiments and elevated somewhat in three experiments. When the values were corrected to a solid content equal to that of the control, a gain of intracellular potassium was indicated in five experiments and an insignificant fall in one. In this connection it is of interest to observe the changes of the intracellular potassium in those animals allowed water but no food for a period of 5 days. In these experiments a gain of intracellular potassium was encountered in every instance, reaching a maximum of 16 milliequivalents in Dog I. It will be observed that while one may conclude that a gain of intracellular potassium of the skeletal muscles of dogs in adrenal insufficiency is frequently encountered it is not as striking as indicated by the observations of others (11).

Following the intraperitoneal injection of a solution containing glucose and potassium chloride, one animal (Dog Y) showed a gain of intracellular potassium, while the other (Dog Z) showed no change. Following the administration of potassium acid phosphate there was no significant change in the intracellular potassium.

*Animals in Adrenal Insufficiency Treated with Cortical Extract—*The blood changes accompanying the clinical improvement of four dogs in adrenal insufficiency following treatment with cortical extract alone were discussed in Paper I (14). It was found that following a period of 48 hours during which cortical extract had

been administered (no food nor water) the concentration of sodium and chloride in the serum had increased with no evidence of blood dilution. It is of interest, therefore, to compare the electrolyte and water contents of skeletal muscles from these four

TABLE V

*Changes of Intracellular Potassium of Dog Muscle*

The values are expressed in terms of 1000 gm. of intracellular water.

Dog	Potas- sium	Solids	Cor- rected potas- sium*	Change	Comment
	m.eq.	gm.	m.eq.	m.eq.	
Con- trol	137	393			
A	113	331	135	-2	Adrenal insufficiency
D	128	338	149	12	" "
E	128	339	149	12	" "
N	149	352	166	29	" "
R	144	363	156	19	" "
S	148	363	160	23	" "
T	129	341	148	11	" " treated
U	139	359	152	15	" " "
V	139	358	153	16	" " "
W	141	357	155	18	" " "
F	137	384	141	4	5 days fasting
G	146	391	147	10	5 " "
I	148	380	153	16	5 " "
K	126	352	141	4	Intraperitoneal glucose
M	126	363	137	0	" "
Q	132	352	147	10	" "
Y	147	375	154	17	" " + KCl
Z	126	365	136	-1	" " "
O	130	385	133	4	High potassium diet
X	137	396	136	-1	" " "

\* The potassium was corrected to an intracellular solid content equal to that of the control.

animals with those reported above for dogs in adrenal insufficiency. The results of the analysis of the skeletal muscles are presented in Table VI. By comparing these data with those given in Table II, it will be observed that no marked changes accompanied the improvement in the animals' condition. Following the treat-

ment with cortical extract the average values for sodium and potassium were slightly higher while that of the chloride was slightly lower than the average levels in insufficiency. From Table V it will be seen that the concentration of potassium in the intracellular water was essentially equal to the control concentration in three experiments and lowered somewhat in one. By

TABLE VI

*Electrolyte and Water Content of Muscle from Dogs in Adrenal Insufficiency Following Cortical Extract Administration*

The values are expressed in terms of 1000 gm. of fat-free muscle.

	Dog T	Dog U	Dog V	Dog W	Average
Chloride, <i>m.eq.</i> .....	15.8	13.2	16.1	14.3	14.9
Sodium, <i>m.eq.</i> .....	12.1	18.5	20.3	20.2	17.8
“ minus chloride, <i>m.eq.</i> .....	-3.7	5.3	4.2	5.9	2.9
Potassium, <i>m.eq.</i> .....	83.8	91.2	90.0	91.8	89.2
Water, <i>gm.</i> .....	780.5	763.5	769.3	767.5	770.2
Extracellular phase, ( <i>F</i> ), <i>gm.</i> .....	139	110	131	120	125
Intracellular water, ( <i>H</i> <sub>2</sub> O) <sub>c</sub> , <i>gm.</i> .....	643	654	640	648	647
Collagen nitrogen, <i>gm.</i> ....	6.14	5.55	5.56	4.86	5.53
$\Delta M^*$ .....	86	6	33	24	37
$\Delta C$ .....	95	54	57	60	66
$\Delta F$ .....	-9	-48	-24	-36	-29
$\Delta' M$ .....	139	31	56	27	63
$\Delta' C$ .....	134	74	72	64	86
$\Delta' F$ .....	5	-43	-16	-37	-23

In these four experiments the muscle samples were obtained 48 hours following the treatment of dogs in adrenal insufficiency with cortical extract alone (no food nor water). For additional discussion see Paper I (14) dealing with the blood changes.

\* See the comments below Table II.

correcting the intracellular potassium concentration to a solid content equal to that of the control, the gains of muscle cell potassium were found to be in the range observed in adrenal insufficiency. Following the treatment with cortical extract there was a slight fall of the total muscle water which appeared to be due to a loss of extracellular water. The calculations of the changes

of the muscle phases relative to the control revealed that an absolute gain in the bulk of the muscle still persisted and was the result of an increase in the intracellular phase and a relatively smaller decrease in the extracellular phase. By comparing the calculated muscle phase changes given in Tables II and VI, it will be observed that the absolute gain in the bulk of the muscle was somewhat less following the treatment with cortical extract. Further, the increase in the intracellular phase was also less, while the decrease in the extracellular phase was somewhat greater. In the light of the accompanying blood changes reported previously one may infer that, following treatment with cortical extract, extracellular fluid had entered the circulation, thus decreasing the magnitude of the extracellular phase. As a result of an accompanying excretion of water (and electrolytes) by way of the urine the concentration of sodium and chloride in the serum became increased. The decrease in the intracellular phase was associated with osmotic adjustments.

The above results lead to the same conclusion as was arrived at previously (14) from studying the electrolyte and water changes of the blood in these animals. That is, they tend to minimize the importance of the correction of the electrolyte and water disturbances as a factor in the part played by the cortical extract in restoring the animals to normal. Such correction is assumed to follow secondarily some other action or actions.

*Analysis of Heart Muscle (Left Ventricle)*—The results of the analysis of heart muscle obtained from several of the animals of the present study are presented in Table VII. To serve as a basis for comparison the average results of the analysis of the left ventricle from five normal dogs are also given. The data were treated in the same manner as those obtained from skeletal muscle. Since only a relatively few observations were made, small changes from the control average are probably without significance. Obviously the calculations for the changes of the muscle phases can be regarded only as approximations. However, they should give some indication of the direction of the changes. By comparing the various experimental values with the average control values, it will be observed that following the intraperitoneal injection of a solution of glucose containing potassium chloride (Dogs Y and Z) there was a tendency for the sodium,

TABLE VII  
*Electrolyte and Water Content of Left Ventricle of Dogs*

The values are expressed in terms of 1000 gm. of fat-free muscle.

	Dog Y	Dog Z	Dog N	Dog S	Dog T	Dog U	Dog V	Dog W	Control
Chloride, <i>m.eq.</i> .....	24.2	25.5	24.7	31.5	24.6	27.3	29.3	29.5	30.4
Sodium, <i>m.eq.</i> .....	23.8	25.5	22.3	26.1	25.9	30.8	26.5	29.5	32.8
Potassium, <i>m.eq.</i> .....	81.8	83.8	79.5	80.3	74.9	81.3	80.8	86.1	77.1
Water, <i>gm.</i> .....	771.5	779.8	786.8	782.8	784.0	783.0	783.0	783.0	785.0
Extracellular phase, ( <i>F</i> ), <i>gm.</i> ...	207	217	221	258	217	227	238	247	249
Intracellular water, ( $H_2O$ ), <i>gm.</i> .....	567	565	568	527	570	558	548	538	539
Muscle cell potassium, <i>m.eq.</i> <i>per kilo intracellular water.</i>	143	146	137	147	129	144	145	158	142
$\Delta M^*$ .....	-72	-27	5	-12	-8	-13	-12	-12	
$\Delta C$ .....	-15	11	32	-18	26	12	2	-7	
$\Delta F$ .....	-57	-38	-27	6	-34	-25	-14	-5	
Serum chloride, <i>m.eq. per kilo</i> <i>water.</i> .....	112.2	112.8	107.1	117.1	109.0	115.2	118.2	114.4	117.2

Dogs Y and Z were studied following the intraperitoneal injection of a solution containing glucose and KCl, Dogs N and S were in adrenal insufficiency, and Dogs T, U, V, and W were studied following treatment with cortical extract alone. The control represents the average from five normal dogs.

\* Calculations were made relative to the control; a constant solid content of the intracellular phase was assumed.

chloride, and water contents to fall. The potassium content appeared to be increased slightly and the potassium concentration per kilo of intracellular water was increased by a small amount (probably an insignificant increase). The calculations of the changes of the muscle phases relative to the control indicated an absolute decrease in the bulk of the muscle, which was chiefly the result of a decrease in the extracellular phase.

In the two animals in adrenal insufficiency (Dogs N and S) the results were somewhat conflicting. Thus, the chloride was lower than the control value in one animal and slightly higher in the other, the sodium tended to be lowered in both, the total water was unchanged, and the potassium content was slightly higher. The potassium concentration per kilo of intracellular water was low in one animal and increased in the other. The calculation of the changes of the muscle phases revealed no uniform direction of change. The analysis of the heart muscles from the four dogs (Dogs T, U, V, and W) treated with cortical extract revealed a slight tendency toward lowered levels of sodium and chloride. The potassium content and the potassium concentration per kilo of intracellular water were slightly above the control average in three experiments and below the average in the fourth. The calculations of the changes of the muscle phases indicated an absolute decrease in the bulk of the muscle, which was due chiefly to a decrease in the extracellular phase.

Attention should be called to the results obtained by Darrow, Harrison, and Taffel (11) on the analysis of heart muscle from animals in adrenal insufficiency. These authors pointed out that the changes in heart muscle are not so regular as those found in skeletal muscle. In the adrenalectomized cat it was found that heart muscle showed a significant increase in water and a questionably significant increase in potassium. In individual experiments the muscle potassium was definitely increased in some animals and lowered in others. It was concluded, therefore, that the increase in cardiac potassium in relation to fat-free solids may develop, but it is not a regular finding in adrenal insufficiency. Their observations indicated that the increase in cardiac water was largely extracellular, there being no evidence of a dilution of the cells. The analyses of the heart muscle from dogs indicated that the changes are like those encountered in the heart muscle of adrenalectomized cats.

The number of observations made in the present study are too few to allow definite conclusions. It is of interest, however, that evidence of a tendency to a contraction of the extracellular phase of the heart muscle was encountered in the animals studied following treatment with cortical extract, since the same change was indicated in the skeletal muscles of these animals.

#### SUMMARY AND CONCLUSIONS

A study has been made of the electrolyte and water content of the skeletal muscle (lumbar portion, sacrospinalis) of twenty-four normal dogs, seven dogs in adrenal insufficiency, and four dogs in adrenal insufficiency following treatment with cortical extract alone. A similar study has also been made on the skeletal muscle from dogs following the intraperitoneal injections of glucose and glucose containing potassium chloride, and following the ingestion of potassium acid phosphate. In addition the results of the analysis of the electrolyte and water content of heart muscle of five normal dogs and eight experimental dogs are presented. The various findings have been discussed and may be briefly summarized as follows:

1. *Content of Normal Dog Muscle*—The analysis of normal skeletal muscle yielded the following results when expressed in terms of 1000 gm. of fat-free tissue, chloride 19.5 milliequivalents  $\pm 2.7$ , sodium 28.2 milliequivalents  $\pm 3.2$ , potassium 83.5 milliequivalents  $\pm 5.5$ , and water 761.4 gm.  $\pm 7.8$ .

Calculations which were made on the basis that the chloride of muscle is extracellular and at a concentration equal to that of an ultrafiltrate of serum gave an extracellular phase ( $F$ ) of 159 gm.  $\pm 22$  per kilo of fat-free muscle and a water content of the intracellular phase of 718 gm.  $\pm 8$  per kilo of muscle cells.

The collagen nitrogen content of normal skeletal muscle (sixteen observations) was found to be 4.88 gm.  $\pm 0.6$  per kilo. By characterizing the extracellular phase of muscle as consisting of extracellular fluid and connective tissue proteins and by making certain assumptions, it was found by calculation that the connective tissue phase of normal muscle amounts to 187 gm.  $\pm 21$  per kilo, containing 84.6 per cent water. As a result of the difference in the magnitude of the extracellular phase, the water content of the intracellular phase was found to be increased to 742 gm.  $\pm 9$  per kilo.

In a number of instances the changes of the muscle phases of the experimental muscles relative to the controls were calculated not only on the basis of the ultrafiltrate chloride concentration alone but also by taking into account the approximate connective tissue contents. Such calculations revealed differences in the magnitude of the changes; however, the directions of the changes were the same and led to the same conclusions.

2. *Skeletal Muscle Changes in Adrenal Insufficiency*—The outstanding changes of the skeletal muscles of dogs in adrenal insufficiency were a loss of sodium and a gain of water. Associated with these changes there was a tendency for the chloride content to fall and the potassium to increase. Evidence that a gain of muscle cell potassium is usually encountered in adrenal insufficiency was presented. However, when this gain was compared with that observed in animals deprived of food for several days, it was not so striking.

The calculations of the changes of the muscle phases relative to the control indicated that in adrenal insufficiency there is an absolute gain in the bulk of the muscle which is due to an increase in the intracellular phase. There was some tendency in the majority of the experiments toward a decrease in the extracellular phase; however, such decrease was of real significance in only two experiments.

It was concluded that in general the results of the present experiments support the concept that the changes of water in the skeletal muscles of dogs in adrenal insufficiency are associated with the electrolyte and water changes of the extracellular fluids.

3. *Skeletal Muscle Changes Following Treatment with Cortical Extract Alone*—No marked electrolyte and water changes of the skeletal muscles from those encountered in adrenal insufficiency accompanied the decided improvement in the animals' condition following treatment with cortical extract alone. The average values for sodium and potassium were slightly higher, while that of chloride was slightly lower than the average levels found in adrenal insufficiency. The gain of muscle cell potassium was in the range observed in adrenal insufficiency. A slight fall in total muscle water was indicated. The calculations of the changes of the muscle phases relative to the control revealed that an absolute gain in the bulk of the muscle still persisted and this was



the result of an increase in the intracellular phase and a smaller decrease in the extracellular phase. Evidence pointed to a greater contraction in the extracellular phase following treatment with the extract than in adrenal insufficiency.

The results of these experiments led to the same conclusion arrived at previously in the study dealing with the blood changes in these animals; namely, they tend to minimize the importance of the correction of the electrolyte and water disturbances as a factor in the part played by the cortical extract in restoring the animal to normal. Such a correction apparently follows secondary to some other action or actions.

4. *Changes in Heart Muscle*—No definite conclusions could be drawn regarding the electrolyte and water changes of heart muscle in adrenal insufficiency, since only two animals were studied and the results were conflicting. Evidence was presented that following treatment with cortical extract there was a tendency toward an absolute decrease in the bulk of the muscle which was apparently due to a decrease in the extracellular phase.

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# ELECTROLYTE AND WATER EQUILIBRIA IN THE DOG

## III. ELECTROLYTE AND WATER EXCHANGE BETWEEN TENDON AND BLOOD

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The electrolyte and water content of the skeletal muscles of normal animals and the changes encountered following various experimental procedures have received considerable attention in recent years. In such studies it has been customary to divide the muscle into extracellular and intracellular phases. In order to estimate the magnitude of the extracellular phase it has been assumed that there is little or no chloride in the muscle cells and that the chloride of muscle is present at a concentration equal to that of an ultrafiltrate of serum (1). Such calculations of the extracellular phase of the skeletal muscles of normal dogs have yielded values which vary from approximately 130 to 190 gm. per kilo of muscle (1, 2), containing 99 per cent water. As a result of the recent studies by Manery (3) and Manery, Danielson, and Hastings (4) it appears that, rather than corresponding to an ultrafiltrate of serum, the extracellular phase is more accurately described as consisting of extracellular fluid and the connective tissue proteins. By taking into account the approximate amount of connective tissue in skeletal muscle, Manery, Danielson, and Hastings (4) found that the extracellular phase of rabbit muscle contains close to 22 per cent solids rather than 1 per cent. The latter authors pointed out that by making such considerations the magnitude of the extracellular phase is increased and the per cent of water in the muscle cells is also increased. In Paper II we (2) attempted to approximate the connective tissue content of the skeletal muscles of normal dogs

and to estimate the changes in the magnitude of the phase by applying the same considerations. The extracellular phase when calculated on the basis of the ultrafiltrate chloride concentration alone amounted to 159 gm., with a standard deviation ( $\sigma$ ) of 22 gm. per kilo of muscle, and the water content of the intracellular phase amounted to 718 gm.,  $\sigma \pm 8$ , per kilo of muscle cells. By taking the approximate connective tissue content into account, we found the magnitude of the phase to be increased to 187 gm.,  $\sigma \pm 21$ , per kilo, containing close to 15 per cent solids, and the water content of the intracellular phase was found to be increased to 742 gm.,  $\sigma \pm 9$ , per kilo of muscle cells.

It is evident that it is desirable to determine any alterations of the connective tissue content when a comparison of experimental with control muscles is made. In Paper II (2) the changes of the experimental muscle phases relative to the control were calculated in a number of instances by taking into account the alterations of the connective tissue content. When the muscle phase changes calculated in this way were compared with those approximated in the usual manner, it was found that the magnitude of the changes differed somewhat; however, the directions of the changes were the same and led to the same interpretations. In such calculations it was assumed, as by Manery, Danielson, and Hastings (4), that the connective tissue of muscle corresponds to tendon. The amount of connective tissue was approximated from determinations of the collagen nitrogen content and the amount of chloride and water it accounts for was based upon the analysis of tendon reported below.

The experiments included in the present report were undertaken for the following purposes: (1) to determine the electrolyte and water content of the tendon from normal dogs, and (2) to observe the changes in tendon accompanying alterations of the electrolyte and water content of the serum.

### *Methods*

The present experiments were carried out in conjunction with the blood and skeletal muscle studies reported in Papers I and II (5, 2) and, with the exceptions of Dogs 1, 2, and 3, the tendon (achilles and quadriceps) was obtained from the same animals. The sampling of the tendon and the methods of chemical analysis

were essentially the same as those described briefly for skeletal muscle (2).

### *Results*

The results of the analysis of the serum and tendon from fifteen dogs for sodium, potassium, chloride, and water are presented in Table I. The results of the first five animals were employed to compute the average values for normal tendon. Dogs O and X were included in this group, since the serum values were within normal limits. The remaining ten dogs represent animals with rather marked electrolyte and water changes of the serum.

From the data presented in Table I it will be observed that, when the values are expressed per kilo of water, the concentrations of the electrolytes in tendon tend to approximate those of serum. By comparing the average values of the serum and tendon from the first five dogs, it will be seen that the serum sodium is 14.5 milliequivalents higher, while the serum chloride and potassium concentrations are 1.3 and 5.6 milliequivalents, respectively, lower than the tendon values. Manery, Danielson, and Hastings (4), who compared serum and tendon from normal rabbits, have already emphasized the similarity of the electrolyte concentrations. Their observations led to the conclusion that the serum influences the chloride and sodium distributions between tendon and serum in the same manner as between an ultrafiltrate and serum. In addition their results indicated that the tendon proteins do not have the same influence on the ionic distributions as the more soluble serum proteins. Thus, when the concentrations of chloride and sodium of tendon were compared, the following average ratios were obtained; namely,  $(\text{Cl})_t/(\text{Cl})_s = 0.88$  and  $(\text{Na})_t/(\text{Na})_s = 0.83$ , which are in reasonable agreement with those obtained between serum and transudates (6). Manery, Danielson, and Hastings (4) found, however, that when the tendon values were calculated from the serum concentrations and the Gibbs-Donnan ratio,  $r = 0.95$ , the tendon chloride exceeded the calculated value in all but one experiment, while the concentrations of tendon sodium were consistently lower than the calculated values. In a more recent study Manery and Hastings (7) examined the electrolyte and water content of various tissues of rats and rabbits and were led to the conclusion that living mam-

TABLE I  
*Electrolyte and Water Content of Dog Serum and Tendon*

The electrolyte values are given in milliequivalents per kilo of water; the H<sub>2</sub>O values in gm. per kilo. The tendon values have been corrected for neutral fat.

Dog No.	Serum					Tendon				$\frac{(Na)_t}{(Na)_s}$	$\frac{(K)_t}{(K)_s}$	$\frac{(Cl)_t}{(Cl)_s}$	Remarks
	Na	K	Cl	H <sub>2</sub> O		Na	K	Cl	H <sub>2</sub> O				
1	153.1	3.3	117.4	917.5		138.6	8.7	121.0	659.8	0.91	2.6	0.97	Normal; mixed diet
2	158.3	3.4	119.4	917.5		144.1		123.3	672.3	0.91		0.97	" "
3	152.8	3.3	113.2	918.3		141.9	6.9	112.8	666.2	0.93	2.1	1.01	" "
O	152.4	4.2	116.7	922.0		139.4	10.9	118.2	658.5	0.91	2.6	0.99	High K diet
X	154.0	3.4	116.0	918.0		134.0	9.9	113.6	684.5	0.87	2.9	1.02	" "
Average....	154.1	3.5	116.5	918.7		139.6	9.1	117.8	668.3	0.91	2.6	0.99	
M	140.9	3.3	102.1	890.5		117.1	5.8	101.9	651.5	0.83	1.8	1.00	Intraperitoneal, 5% glucose
Q	141.7	3.2	100.1	881.0		120.4	10.6	104.1	633.0	0.85	3.3	0.96	" "
Y	153.5	4.8	112.2	871.5		121.9	14.1	114.8	638.5	0.79	2.9	0.98	" "
Z	146.7	5.7	112.8	888.5		129.6	11.2	117.2	636.5	0.88	2.0	0.96	KCl Intraperitoneal 5% glucose +
N	140.2	9.1	107.1	895.5		126.8	14.2	117.6	649.8	0.90	1.6	0.91	KCl Adrenal insufficiency
S	138.5	11.1	117.1	882.5		136.8	9.1	114.8	652.5	0.99	0.8	1.02	" "
T	137.2	8.2	109.0	903.5		118.7	17.4	113.8	661.8	0.86	2.1	0.96	" treated
U	147.7	4.5	115.2	887.5		130.0	13.9	122.8	642.5	0.88	3.1	0.94	" "
V	143.2	6.9	118.2	895.5		132.0	15.5	116.2	665.8	0.92	2.2	1.02	" "
W	140.9	5.6	114.4	895.3		128.1	15.0	121.3	654.0	0.91	2.7	0.94	" "
Average....	143.1	6.2	110.8	889.1		126.1	12.7	114.5	648.6	0.88	2.3	0.97	

malian tissues cannot be divided into intra- and extracellular phases in the same manner as muscle. Their observations indicated that for the purposes of an approximate description of tissues at least three chemically distinct phases are necessary: (1) an extracellular phase,  $(E)_p$ , which is in ionic equilibrium with blood plasma and consists essentially of plasma ultrafiltrate and connective tissue proteins; (2) an intracellular phase,  $(C)_1$ , which contains neither sodium nor chloride and is exemplified by muscle fibers; and (3) an intracellular phase,  $(C)_2$ , which contains chloride and may or may not have sodium in equivalent proportions. In regard to tendon it was concluded (4, 7) that it consists almost entirely of the extracellular phase,  $(E)_p$ . However, in view of the relative differences of the concentrations of chloride and sodium indicated above, it was concluded that there is present in addition a small intracellular phase,  $(C)_2$ , which is relatively high in chloride and low in sodium. Evidence for the latter is also found in the observation that the potassium concentration of tendon is at least twice that of serum and the excess of potassium might be associated with this phase. Amberson, Nash, Mulder, and Binns (8) also concluded that some of the chloride of tendon must be intracellular, since the calculated chloride space was found to exceed the determined amount of water.

From Table I it will be observed that the average concentration ratios for sodium and chloride between serum and tendon in normal dogs, namely,  $(Na)_t/(Na)_s = 0.91$  and  $(Cl)_t/(Cl)_s = 0.99$ , agree rather well with those which have been reported for serum and transudates (6). Since the chloride ratios are above and the sodium ratios are below the Gibbs-Donnan ratio of 0.95, it is evident that the observed tendon concentrations in each case would be somewhat less than the corresponding concentrations calculated from the serum values and the ratio 0.95. It will be noted that the concentration of potassium in dog tendon is more than twice that of serum; *i.e.*,  $(K)_t/(K)_s = 2.6$ . The explanation for this relative excess of potassium in tendon is not entirely clear; however, it might be attributed to the presence of cells relatively devoid of sodium and chloride. When the gm. of water per kilo of tissue is calculated from the average values for the tendon chloride and the ultrafiltrate chloride concentration, a value of 642 gm. is obtained ( $78.7 \times 0.95 \times 1000/116.5 = 642$ ).



Thus, in contrast with the observation of Amberson *et al.* (8), who studied the tendon from cats, the calculated water content is somewhat less than the determined values.

From the results of the present experiments it appears unnecessary to assume the presence of an intracellular phase in dog tendon which contains relatively more chloride than sodium. Since the concentrations of sodium and chloride in dog tendon are at the levels one would anticipate for a simple ultrafiltrate of serum, it appears that the tendon may be described as consisting chiefly of connective tissue proteins diluted with serum ultrafiltrate.

By comparing the average values for the two groups of animals presented in Table I, it will be seen that in general the changes in serum were associated with parallel changes in the tendon. Thus, it will be observed that with an average fall of the serum water of 29.6 gm. per kilo there was an average decrease of the tendon water of 19.7 gm. per kilo. The tendon water calculated from the average values for tendon chloride and the ultrafiltrate chloride concentration amounts to 638 gm. per kilo ( $74.4 \times 0.95 \times 1000/110.8 = 638$ ), compared with the calculated value of 642 gm. for normal tendon. This apparent decrease of the tendon water is of some interest, since in the skeletal muscles of these animals (2) a tendency toward a decrease in the magnitude of the extracellular phase was noted. (Some tendency toward a decrease in the magnitude of the extracellular phase of the skeletal muscles of Dogs O and X was also indicated (2).) With a fall of the sodium and chloride concentrations of the serum there appeared to be a relatively greater loss of sodium than chloride from tendon. Thus, the average ratio  $(Na)_t/(Na)_s$  was found to be 0.88, while that for chloride,  $(Cl)_t/(Cl)_s$ , was found to be 0.97. Whereas the finding was not entirely regular, there was an evident tendency for the concentration of potassium in tendon to be increased when the serum potassium concentration was raised.

It will be observed that on the whole the various distribution ratios obtained in these animals do not differ greatly from those observed with the normal animals. Therefore, within limits, it appears that the changes of the electrolyte concentrations of dog tendon accompanying changes in the serum are such as to establish ionic (Donnan) equilibrium.

## SUMMARY AND CONCLUSIONS

The tendons from five dogs with normal serum electrolyte patterns and from ten dogs with rather marked serum electrolyte and water changes have been analyzed for sodium, potassium, chloride, and water. The results have been discussed briefly and may be summarized as follows:

1. The tendon from normal dogs yielded the following average results when expressed in terms of a kilo of fat-free tissue: sodium 3.4 milliequivalents, potassium 6.1 milliequivalents, chloride 8.7 milliequivalents, and water 668.3 gm.

2. The average concentration ratios for sodium and chloride between serum and tendon in normal dogs were found to be  $(Na)_t/(Na)_s = 0.91$  and  $(Cl)_t/(Cl)_s = 0.99$ . Since the concentrations of sodium and chloride of tendon were essentially equal to those of an ultrafiltrate of serum, it was concluded that there is little evidence for the presence of an intracellular phase in dog tendon which is relatively high in chloride and low in sodium. The average potassium concentration ratio of  $(K)_t/(K)_s = 2.6$  was taken to indicate the presence of a small intracellular phase which contains potassium but which is relatively devoid of sodium and chloride. It was concluded that dog tendon may be described as consisting chiefly of connective tissue proteins diluted with serum ultrafiltrate.

3. Within limits, it appeared that the changes of the electrolyte concentrations of dog tendon accompanying changes in the serum are such as to establish ionic (Donnan) equilibrium.

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# THE INFLUENCE OF STRUCTURE ON THE ELIMINATION MAXIMUM

## I. THE STRUCTURE OF VITAMIN A<sub>2</sub>\*

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Previous work has indicated that at very low pressures, 10  $\mu$  or less, the addition of one CH<sub>2</sub> group to a compound causes a rise of 5° in the elimination maximum (1). In other words, the elimination maximum of each member of a homologous series is believed to be 5° higher than the one preceding it. The precession of elimination maxima is of some value in vitamin research and we may consider it now in connection with vitamin A. Gillam *et al.* (2) have postulated that vitamin A<sub>2</sub> is a higher homologue of vitamin A, containing 2 more carbon atoms and one more double bond. One of us (3) has compared the elimination maxima of the two vitamins, and has found that the maximum of vitamin A<sub>2</sub> is only 3° higher than that of A, suggesting that the two do not differ by as much as 2 carbon atoms. However, since the elimination maximum of vitamin A<sub>2</sub> is 3° higher than that of A, it is of interest to determine whether the difference can be accounted for by the additional conjugated double bond postulated by Gillam *et al.* We have, therefore, extended our investigations to include the effect of various types of unsaturation on the elimination maximum.

The determinations were carried out with fatty acids for the following reasons, (1) they have elimination maxima in the same region as vitamins A and A<sub>2</sub>; (2) they are easily estimated by titration in a neutral oil such as synthetic constant yield oil (4); (3) sufficient members of the series are available or can be prepared to make the desired comparisons. Lauric, myristic, pal-

\* Communication No. 17 from the Laboratories of Distillation Products, Inc.

mitic, stearic, oleic, linoleic, 9,11-linoleic, and  $\alpha$ -eleostearic acids were included in the series.

A note on the effect of impurities on the elimination maxima of the acids should be included at this point. The contaminating acid may be either higher or lower in the series than the acid under investigation. It was found that there is a linear relationship between the amount of impurity and the shift in elimination maximum. An example may be cited. Stearic acid has a maximum at  $118^\circ$ , while that of palmitic acid is  $108^\circ$ . A mixture of 90 per cent stearic acid and 10 per cent palmitic acid is found to have a maximum at  $117^\circ$ . Since the limit of error is  $\pm 1^\circ$ , this 10 per cent impurity would scarcely cause a significant alteration in the maximum. Obviously if the maxima of the two substances in the mixture were less than  $10^\circ$  apart, the alteration in the maximum would be less than  $1^\circ$ , still on the assumption of the 9:1 ratio.

#### EXPERIMENTAL

##### *Materials*

Stearic acid, m.p.  $69^\circ$ , palmitic acid, m.p.  $61\text{--}61.5^\circ$ , and lauric acid, m.p.  $43\text{--}43.5^\circ$ , were obtained from the Eastman Kodak Company. Myristic acid as received from both the Eastman Kodak Company and the Pfanstiehl Chemical Company gave maxima  $3^\circ$  or  $4^\circ$  below theory. However, the Pfanstiehl sample recrystallized twice from ethanol-water, m.p.  $52\text{--}53^\circ$ , gave the expected maximum. Oleic acid, m.p.  $13^\circ$ , iodine No. 89.0, and linoleic acid, m.p.  $-7^\circ$ , iodine No. 183.0, were prepared from the acids of olive oil and California walnut oil, respectively, by the methods of Brown and coworkers (5, 6).  $\alpha$ -Eleostearic acid, m.p.  $45\text{--}46^\circ$ , was prepared from tung oil. 9,11-Linoleic acid, m.p.  $53^\circ$ , iodine No. 129.8,  $E_{1\text{ cm}}^{1\%}$  (230  $m\mu$ ) equal to 992 in absolute ethanol, was prepared by distilling crude ricinelaidic acid in the presence of anhydrous  $\text{Al}_2\text{O}_3$  at 30 mm. (7). According to van der Hulst (8),  $E_{1\text{ cm}}^{1\%}$  is equal to 1200 for the pure acid.

##### *Method*

3 gm. of the acid were dissolved in 50 ml. of constant yield oil and 150 ml. of corn oil residue,<sup>1</sup> and distilled analytically (9) in a

<sup>1</sup> Commercial corn oil from which the volatile constituents have been removed, leaving only the higher triglycerides.

500 ml. cyclic molecular still, with a 10 minute cycle and  $10^\circ$  temperature intervals. For analysis, each fraction was dissolved in 20 ml. of neutralized ethanol and titrated against phenolphthalein with 0.1 N aqueous NaOH. In order to avoid crystallization of the higher melting acids, with attendant chance of loss and error, cooling of the condenser was dispensed with. Repeated

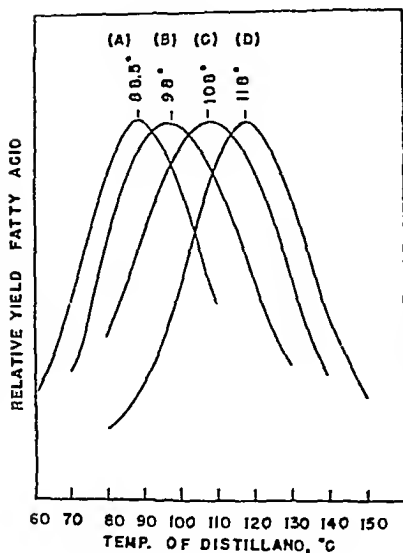


FIG. 1

FIG. 1. Elimination maxima of saturated fatty acids. Curve A, lauric acid; Curve B, myristic acid; Curve C, palmitic acid; Curve D, stearic acid. The temperature indicated on each curve is the elimination maximum of that curve.

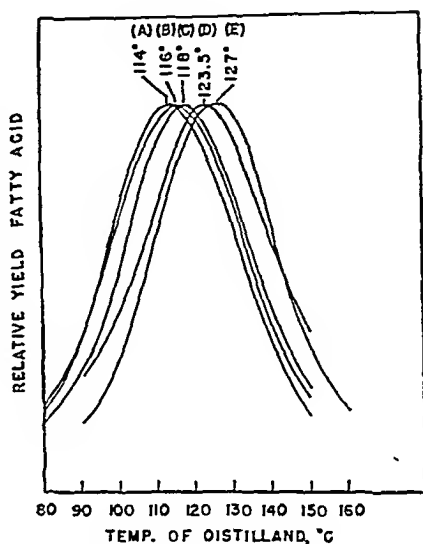


FIG. 2

FIG. 2. Elimination maxima of various unsaturated acids as compared to stearic acid. Curve A, linoleic acid; Curve B, oleic acid; Curve C, stearic acid; Curve D, 9,11-linoleic acid; Curve E,  $\alpha$ -eleostearic acid. The temperature indicated on each curve is the elimination maximum of that curve.

distillations of linoleic acid both with and without this cooling showed that no error was introduced, the total deviation being  $1^\circ$ .

#### DISCUSSION

The elimination curves and maxima are given in Figs. 1 and 2. Fig. 1 shows a comparison between the straight chain saturated

acids  $C_{12}$ ,  $C_{14}$ ,  $C_{16}$ , and  $C_{18}$ . As may be seen, there is a  $10^\circ$  temperature rise between successive acids, corresponding to  $5^\circ$  for each  $CH_2$  group. In Fig. 2 is shown the effect of unsaturation upon the maximum. A group of unsaturated  $C_{18}$  acids is compared with stearic acid. Unsaturation without conjugation lowers the maximum  $2^\circ$ ; e.g., oleic acid is  $2^\circ$  below stearic acid, and linoleic acid is  $2^\circ$  below oleic. However, when the double bonds are in conjugation, the maximum is raised. Thus  $\alpha$ -eleostearic acid has a maximum  $9^\circ$  higher, and 9,11-linoleic acid  $6^\circ$  higher, than stearic acid. Since eleostearic acid has three conjugated double bonds, and since its maximum is approximately  $3^\circ$  higher than 9,11-linoleic acid with two conjugated double bonds, the additional conjugated double bond has raised the maximum  $3^\circ$ . Preliminary work on sterols indicates that an additional conjugated double bond in a ring raises the elimination maximum in the same manner as does one in a chain. This agrees with the findings for vitamins A and  $A_2$ . As vitamin  $A_2$  has one more conjugated double bond than vitamin A, and its maximum is  $3^\circ$  higher, it probably has the same number of carbon atoms as vitamin A.

#### SUMMARY

1. A comparison of the elimination curves of lauric, myristic, palmitic, and stearic acids has confirmed the postulate that one  $CH_2$  group raises the elimination maximum  $5^\circ$ .

2. A comparison of the elimination curves of oleic, linoleic, and stearic acids shows that each non-conjugated double bond lowers the maximum  $2^\circ$ . However, when the double bonds are conjugated, each additional double bond raises the maximum  $3^\circ$ , as shown by a comparison of the curves of stearic, 9,11-linoleic, and  $\alpha$ -eleostearic acids.

3. Our observations are in accord with the view that vitamin  $A_2$  contains the same number of carbon atoms as vitamin A but differs in that it has one additional conjugated double bond.

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# CHEMICAL STUDIES OF CERTAIN PATHOGENIC FUNGI

## III. FURTHER STUDIES ON THE LIPIDS OF BLASTOMYCES DERMATIDITIS AND MONILIA ALBICANS

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The present report is a continuation of studies carried out in this laboratory on the chemistry of the lipids of two pathogenic yeast-like fungi, *Blastomyces dermatiditis* and *Monilia albicans*. The previous reports (1) dealt with the lipids extracted by alcohol, ether, and chloroform at room temperature. These lipids, making up about 10 per cent of the cells of *Blastomyces dermatiditis* and 5 per cent of *Monilia albicans*, were found to be mixtures of lecithin, cephalin, glycerides, free fatty acids, and sterols. The present report is concerned with the lipids yet remaining in the cells of these organisms after the above extractions.

From both organisms the remaining lipids were extracted in three fractions: Fraction I, by boiling neutral alcohol; Fraction II, by boiling solvents containing hydrolytic reagents; and Fraction III, by ether, after the cell residues were dissolved in hydrochloric acid. Fractions II and III represent firmly bound lipids. These three fractions together formed 5.7 per cent of the cells of *Blastomyces dermatiditis* and 8.6 per cent of *Monilia albicans*.

The corresponding fractions isolated in the present study from both fungi were closely similar in composition. Fraction I in both cases was a mixture which could be separated into three parts by means of differences in solubility. These were Fraction I-a, a white amorphous powder having a nitrogen to phosphorus ratio of about 2:1, evidently a phosphatide; Fraction I-b, a buff-colored amorphous powder containing about 7 per cent of nitrogen and considerable polysaccharide but only traces of phosphorus; and Fraction I-c, a brown oil yielding sterols, glycerol, and

palmitic, stearic, oleic, and linoleic acids on saponification. Fraction II from both fungi was a brown oil similar to Fraction I-c and yielding the same substances on saponification. Traces of carbohydrates were also observed in the saponification mixtures from Fraction II. It is possible that polysaccharides originally formed a part of the bound lipids but were split off as a result of the hydrolytic extraction procedures. Incompleteness of such splitting would account for the traces of polysaccharides found among the saponification products of the lipids. Fraction III from both organisms was a pale yellow oil consisting of fatty acids and sterols. This fraction represents only the ether-soluble part of the remaining bound lipid material. The isolation of the bound lipids of these organisms in a relatively unchanged state remains a problem for future study.

It should be mentioned that the fungus-bound lipids which were isolated as an oil differed distinctly from the bound lipids of the acid-fast group of bacteria studied by Anderson and co-workers (2). The latter substances were white powders yielding hydroxy acids of high molecular weight and specific polysaccharides on saponification. In appearance at least, the bound lipids of the fungi resembled those of *Phytomonas tumefaciens* reported by Geiger and Anderson (3).

#### EXPERIMENTAL

The cells of *Blastomyces dermatiditis* and *Monilia albicans* used in the following experiments had been thoroughly extracted at room temperature with alcohol-ether mixtures and chloroform and dried *in vacuo* over sulfuric acid. The cells were then ground in a ball mill.

In all operations dealing with unsaturated lipids, carbon dioxide or nitrogen atmospheres were maintained where possible. Ether and alcohol were distilled over potassium hydroxide.

*Extraction of Lipids*—A 36.32 gm. sample of *Blastomyces dermatiditis* cells dried to constant weight over phosphorus pentoxide was placed in a 1 liter bolthead flask equipped with a reflux condenser attached by a ground joint. The sample was then refluxed successively four times with alcohol. The extracts were filtered off while still warm. The cells were washed once with warm alcohol, then quantitatively transferred back into the ex-

traction flask with the aid of the succeeding lot of solvent, and refluxing was begun again. The four extracts were combined and treated as described below, yielding Fraction I. A subsequent extraction was made with boiling ether.

The cells were then successively extracted in the same manner with alcohol containing 1 per cent of hydrochloric acid, alcohol-ether (1:1) containing 1 per cent of hydrochloric acid, and alcohol containing 2 per cent of potassium hydroxide. These three extracts were saved and treated as described below, yielding Fraction II.

After the above extractions, the cells were dried and 0.5 to 1.0 gm. samples were dissolved in hydrochloric acid and extracted with ether, according to the method described by Turpeinen (4). The ether extracts were washed with water, dried, and evaporated, yielding Fraction III.

*Isolation of Lipids*—In the combined alcohol extracts of the cells, a precipitate had formed on cooling to room temperature. After standing 2 days in the refrigerator, this precipitate was centrifuged off, washed with alcohol, and dried. In this manner there was obtained 0.106 gm. of a white powder, Fraction I-a, which formed 0.29 per cent of the cells. The supernatant solution and washings from the isolation of Fraction I-a were concentrated to dryness under reduced pressure, and extracted with ether. An insoluble residue remained, part of which was soluble in warm absolute alcohol. On addition of 2 volumes of acetone to the alcohol extract, a buff-colored precipitate, Fraction I-b, was obtained. This was reprecipitated in the same way and when dried weighed 0.035 gm., 0.10 per cent of the cells. The ether extract mentioned above was washed with water, dried, and evaporated, leaving a dark brown oil weighing 0.8450 gm., 2.33 per cent of the cells. This was designated Fraction I-c.

The ether extract of the cells was evaporated, leaving 0.030 gm. of a brown oil. This was combined with Fraction I-c.

The acid alcohol extracts were neutralized and concentrated to small volume under reduced pressure. Water was added and the lipids were extracted with ether. The ether extract was washed with water, dried over sodium sulfate, and evaporated, leaving 0.2130 gm. of a brown oil, 0.59 per cent of the cells. This was called Fraction II-a. The acid alcohol-ether extracts treated

in the same manner yielded 0.0768 gm. of brown oil, 0.21 per cent of the cells. This was called Fraction II-b. The alkaline alcohol extract was concentrated to small volume under reduced pressure. Water was added and unsaponifiable lipid material was extracted with ether. After removal of unsaponified lipids, the water solution was acidified with hydrochloric acid, and fatty acids were extracted with ether. The two ether extracts were washed and dried in the usual manner and yielded on evaporation 0.0103 gm. of fatty acids and 0.0241 gm. of non-saponified material. This material, Fraction II-c, formed 0.09 per cent of the cells.

The ether extracts of the cell residues dissolved in hydrochloric acid were washed with water, dried over sodium sulfate, evaporated, and weighed. The residue in each case was a pale yellow

TABLE I  
*Fractions of Lipids Isolated from Blastomyces dermatiditis and Monilia albicans*

Fraction No.	<i>Blastomyces dermatiditis</i>		<i>Monilia albicans</i>	
	gm.	per cent	gm.	per cent
I	1.016	2.80	0.9983	3.42
II	0.3242	0.89	0.3562	1.22
III	0.7264*	2.00	1.168*	4.00
Total.....	2.0666	5.69	2.5225	8.64

\* Calculated on the basis of determinations on small samples.

oil, Fraction III. Three determinations were made on the cell residues of each sample and the results were averaged.

Table I summarizes the data on yields of the three fractions.

A 29.19 gm. sample of *Monilia albicans* cells was treated as described for the sample of *Blastomyces dermatiditis* cells. The yields of the three fractions are given in Table I.

The weights and percentages of the subfractions from *Monilia albicans* were as follows: Fraction I-a, 0.277 gm., 0.95 per cent; Fraction I-b, 0.057 gm., 0.20 per cent; Fraction I-c, 0.6643 gm., 2.28 per cent; Fraction II-a, 0.2230 gm., 0.76 per cent; Fraction II-b, 0.0954 gm., 0.33 per cent; and Fraction II-c, 0.0378 gm., 0.13 per cent.

Duplicate samples of both *Blastomyces dermatiditis* and *Monilia*

*albicans* cells were extracted as described above. The results agreed very closely with those in Table I. The amounts of the various subfractions obtained also agreed well with the percentage reported here.

*Examination of Fractions*—Fraction I-a from both organisms was a white powder, soluble in hot alcohol. After reprecipitation from this solvent, the substance was in each case analyzed for nitrogen and phosphorus.

*Microanalysis*—

Fraction I-a from

*Blastomyces dermatiditis*. Found. N 3.00, P 2.55, N:P 2.6

*Monilia albicans*. " " 1.21, " 1.38, " 1.9

When stirred up in water, the substance from *Blastomyces dermatiditis* gave a positive Molisch test for carbohydrate. However, the powder was practically insoluble in water and in acid or alkaline aqueous solutions. The substance was somewhat soluble in pyridine, going very slowly into solution. From pyridine solution it could be completely precipitated by the addition of 1 volume of cold acetone. In chloroform-methyl alcohol mixture (1:1), in hot acetone, and in warm ether the substance was slightly soluble. When stirred up in carbon tetrachloride and treated with bromine, the substance absorbed a small amount of bromine, indicating the presence of unsaturation. Saponification with alcoholic potassium hydroxide yielded fatty acids and water-soluble material. During the saponification, an insoluble substance appeared on the walls of the flask. This was found to be a carbohydrate. The water-soluble part consisted of carbohydrates and nitrogenous substances which have not been identified. Fraction I-a from *Monilia albicans* showed essentially the same properties as Fraction I-a from *Blastomyces dermatiditis*.

Fraction I-a from both fungi thus appeared to be a phosphatide possessing properties somewhat similar to those of sphingomyelin. Insufficient material was available for further study. Traces of this material were observed in the acid alcohol extracts of both fungi.

Fraction I-b from *Blastomyces dermatiditis* was a buff-colored powder, partially soluble in water. It gave a positive Molisch reaction for carbohydrate. When stirred up in carbon tetra-

chloride and treated with bromine, it absorbed no bromine, indicating that it was a saturated compound. Saponification showed the presence of fatty acids, a polysaccharide, and nitrogenous compounds. The corresponding fraction from *Monilia albicans* possessed like properties.

*Microanalysis—*

Fraction I-b from

*Blastomyces dermatiditis*. Found. N 7.5, P traces

*Monilia albicans*. " " 7.0, " "

Fraction I-c from both organisms was a brown oil containing only traces of phosphorus and nitrogen. It was saponified in

TABLE II

*Fatty Acids and Unsaponifiable Material in Lipid Fractions of Blastomyces dermatiditis and Monilia albicans*

	Fraction No.	Sample	Fatty acids isolated		Unsaponifiable material isolated	
		gm.	gm.	per cent	gm.	per cent
<i>Blastomyces dermatiditis</i>	I-c	0.8442	0.3080	36.5	0.4940	58.5
	II-a	0.2100	0.0643	30.6	0.1380	65.7
	II-c		0.0103	29.9*	0.0241	70.1*
	Mixed bound lipids	0.6000	0.2396	39.9	0.3284	54.7
<i>Monilia albicans</i>	I-c	1.688	1.015	60.1	0.3920	23.2
	II-a	0.2200	0.1157	52.6	0.0968	44.0
	II-c		0.0082	21.7*	0.0296	78.3*

\* Calculated on the basis of total ether extract.

both cases by refluxing for 7 hours with 4 per cent alcoholic potassium hydroxide. After removal of most of the alcohol and dilution with water, the unsaponifiable material was extracted with ether. The ether extract was washed with water and dried over sodium sulfate. The ether was removed and the unsaponifiable material weighed. The aqueous solution containing the soaps of the fatty acids was acidified with hydrochloric acid and extracted with ether. The ether extract was washed with water, dried over sodium sulfate, and evaporated to dryness. The residue of fatty acids was weighed. The results are summarized in Table II.

Fractions II and III represent bound lipids, since some hydrolysis was necessary before they could be removed. Fractions

II-a and II-b from both fungi were brown oils similar in appearance to Fraction I-c. They were saponified in the same manner, and the results appear in Table II. Since the extraction of Fraction II-c actually saponified this fraction, the data on the amounts of fatty acids and unsaponifiable material obtained are also included in Table II. A sample of mixed bound lipids obtained from *Blastomyces dermatiditis* in another experiment was saponified as described above. The data are given in Table II.

The data in Table II show an unusually large percentage of unsaponifiable material in the bound lipid fractions of both *Blastomyces dermatiditis* and *Monilia albicans*. In almost every case, there is considerably more unsaponifiable material than in the lipids extracted with neutral solvents. The percentage of fatty acids is correspondingly lower in the bound lipids than in the easily extracted lipids. These results are not due to incomplete saponification of the bound lipid fraction, for resaponification of the combined unsaponifiable fractions gave no further yields of fatty acids.

**Fatty Acids**—All of the crude fatty acid fractions isolated were brown semicrystalline masses. The acids from Fraction I of *Blastomyces dermatiditis* and the acids from the mixed bound lipids of this organism were titrated in neutral alcohol with 0.1 N potassium hydroxide, giving neutral equivalents of 271 and 273, respectively. The corresponding fractions from *Monilia albicans* gave neutral equivalents of 274 and 276. In view of the similarity in neutral equivalents, the acids of all three fractions of each organism were worked up together. Separation of the mixed acids of *Blastomyces dermatiditis* into saturated and unsaturated fractions by the Twitchell (5) method gave approximately 45 per cent of saturated acids and 55 per cent of unsaturated acids. Similar treatment of the mixed acids from *Monilia albicans* lipids gave approximately 40 per cent of saturated acids and 60 per cent of unsaturated acids. The saturated acids of both organisms appeared to be mixtures of palmitic and stearic acids. All of the unsaturated acid fractions from the two organisms had iodine numbers between 90 and 100, as determined by the Rosenmund-Kuhnhehn method (6). On bromination of these unsaturated fractions in cold light petroleum ether, very small amounts of insoluble material, evidently tetrabromostearic acid, were ob-



tained. This insoluble bromide from the unsaturated fractions of both fungi melted at 111–113°, but insufficient material was available for recrystallization or analysis. The main product of bromination in both cases was a pale brown oil, probably dibromostearic acid. The data indicate that oleic and linoleic acids make up the unsaturated fractions of the fatty acids from the alcohol-soluble lipids and the bound lipids of both fungi.

*Unsaponifiable Material*—The unsaponifiable fractions of all the lipid extracts described in this report were brown oils from which white crystals separated on standing. All of these fractions gave qualitative tests indicating the presence of sterols. Colorimetric sterol determinations were made by the Bloor (7) method. With ergosterol as standard, 20.4 per cent of sterols in the mixed unsaponifiable material from Fractions I and II of *Monilia albicans* was found, and 8.4 per cent in the unsaponifiable material from Fractions I and II of *Blastomyces dermatiditis*. Calculated on the basis of total lipids isolated, this corresponded respectively to 0.24 per cent and 0.40 per cent of the cells of the two fungi.

In a separate experiment, 63 gm. of *Monilia albicans* cells, previously extracted with alcohol and ether, were extracted overnight at room temperature with 300 cc. of 2 per cent potassium hydroxide in 25 per cent alcohol. The suspension was centrifuged and the supernatant solution saved. The moist cells were suspended in 500 cc. of 95 per cent alcohol containing 4 per cent potassium hydroxide, and kept at 40° for 7 hours. The cells were then filtered off and washed with alcohol. Filtrate and washings were combined with the supernatant solution from the 25 per cent alcohol extract which had been treated with 2 volumes of alcohol and centrifuged to remove polysaccharides. The combined solutions were concentrated under reduced pressure to small volume, diluted with water, and extracted with ether. The ether extract was dried over sodium sulfate and evaporated, yielding 0.294 gm. of unsaponifiable material. When this was dissolved in 25 cc. of hot 80 per cent alcohol and treated with an excess of digitonin, there was obtained 0.442 gm. of digitonide, corresponding to about 0.110 gm. of sterol (0.18 per cent of the cells). After the unsaponifiable material was removed, the saponification mixture was acidified. The fatty acids which were isolated in the usual manner weighed 4.995 gm. (7.93 per cent of cells).

In a similar experiment 36.3 gm. of *Blastomyces dermatiditis* cells, previously extracted with alcohol and ether, were extracted with 500 cc. of 4 per cent potassium hydroxide in 95 per cent alcohol for 7 hours at 40°. The suspension was filtered while warm, and the cells washed with alcohol. The extract and washings were concentrated and the lipids isolated as described above. There were obtained 73 mg. of unsaponifiable material which yielded 38 mg. of digitonide corresponding to about 9 mg. of sterol (0.03 per cent of cells). The fatty acids isolated weighed 34 mg. (0.09 per cent of cells). Subsequent treatment with 300 cc. of alkaline 25 per cent alcohol and reextraction with 500 cc. of 4 per cent potassium hydroxide in 95 per cent alcohol removed only 25 mg. more of lipid material.

*Water-Soluble Material*—The presence of glycerol in the water-soluble portions of all of the lipid fractions was indicated by the acrolein test. In the case of the combined water-soluble portions of the *Blastomyces dermatiditis*-bound lipids, neutralization, evaporation to dryness, and extraction with absolute alcohol yielded a small amount of thick brown syrup. Benzoylation of this syrup by the method of Einhorn and Hollandt (8) yielded glycerol tribenzoate, melting at 75–76°. When mixed with authentic glycerol tribenzoate, there was no depression of the melting point. The presence of glycerol in the bound lipids of *Blastomyces dermatiditis* is thus established. A positive Molisch test was given by all the water-soluble portions, indicating the presence of at least traces of carbohydrates.

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#### SUMMARY

1. The two pathogenic fungi, *Blastomyces dermatiditis* and *Monilia albicans*, after thorough extraction at room temperature with alcohol, ether, and chloroform, still contained respectively 5.7 and 8.6 per cent of lipid material. This was partly removed by boiling alcohol. The remaining lipid material, the bound lipids, was removed by hydrolytic extraction procedures.
2. The hot alcohol extracts of both fungi contained (1) a phosphatide with an N:P ratio of about 2:1, (2) a nitrogenous lipid containing about 7 per cent of nitrogen, and yielding fatty acids

and polysaccharides on saponification, and (3) an oil yielding fatty acids, sterols, and glycerol on saponification.

3. The bound lipids of both organisms were brown oils which appeared to contain palmitic, stearic, oleic, and linoleic acids, glycerol, and sterols.

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## ON THE SYNTHESIS OF SERINE

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In a recent communication Schiltz and Carter (1) have pointed out the difficulties encountered in the synthesis of hydroxyamino acids including serine, and have offered a new synthesis for the latter based on the formation of methyl  $\alpha$ -bromo- $\beta$ -methoxypropionate. The latter compound was formed by the addition of methyl alcohol and mercuric acetate to methyl acrylate. It occurred to us that this intermediate or a similar one could be prepared for this purpose by the reaction of sodium methylate or ethylate with the methyl or ethyl ester of  $\alpha,\beta$ -dibromopropionic acid and thus the use of mercury salts could be circumvented. The work of Michael (2) with ethyl dibromopropionate led us to believe that this method of approach to the synthesis of serine might be feasible. The dibromo esters can of course be readily prepared by the bromination of methyl or ethyl acrylate, both of which are now commercially available.

In his studies on ethyl  $\alpha,\beta$ -dibromopropionate Michael found that an ethoxy bromopropionate was produced by the action of 1 mole of sodium ethylate upon the dibromo ester. There was some question, however, as to the position of the ethoxy group. Later the same ethoxy compound was obtained by the addition of sodium ethylate to ethyl  $\alpha$ -bromoacrylate, indicating that the compound derived from the ethyl  $\alpha,\beta$ -dibromopropionate must have been the  $\beta$ -ethoxy compound. An excess of the sodium ethylate was shown by Michael to lead to the formation of a halogen-free compound, while Leighton (3) found that heating the dibromo ester with an excess of sodium ethylate resulted in the formation of a mixture of products which were apparently the esters of  $\beta$ -ethoxyacrylic and  $\alpha,\beta$ -diethoxypropionic acids.

In studying the action of sodium ethylate with ethyl  $\alpha,\beta$ -dibromopropionate we therefore avoided an excess of sodium ethylate and carried out the reaction at a low temperature. By using equimolecular quantities of the two substances at  $0^\circ$  we obtained a yield of 80 to 85 per cent of the  $\alpha$ -bromo- $\beta$ -ethoxypropionate. A convenient and inexpensive synthesis of serine was thus assured.

The ethyl  $\alpha$ -bromo- $\beta$ -ethoxypropionate was saponified and converted to serine by the general procedure employed by Carter and West (4) for  $\alpha$ -bromo- $\beta$ -methoxypropionic acid.<sup>1</sup> The ethoxy compound was utilized in the present synthesis, since it was found that when sodium methylate was allowed to react with the dibromo ester much poorer yields of the methoxy derivative were obtained owing to the side reactions which occurred.

#### EXPERIMENTAL

Ethyl acrylate was placed in a round bottomed flask immersed in an ice bath. The theoretical quantity of bromine was added dropwise with stirring. 15 minutes after the addition was completed the product was distilled under reduced pressure. Practically a quantitative yield of ethyl  $\alpha,\beta$ -dibromopropionate was obtained. The boiling point of the pure compound was  $98.5-99.5^\circ$  at 16 mm. of Hg. Experiments in which amounts up to 4 moles of ethyl acrylate were used yielded similar results.

260 gm. of the ethyl  $\alpha,\beta$ -dibromopropionate were placed in a 1 liter, 3-necked flask equipped with drying tube, dropping funnel, and mechanical stirrer. 24 gm. of clean sodium metal were dissolved in 400 cc. of 100 per cent ethanol with rigid exclusion of all moisture. This solution was slowly added to the ester, the mixture being vigorously stirred and kept at ice bath temperature. Precipitation of the sodium bromide was immediate. At the end of the addition the ice bath was removed and the suspension was stirred until it attained room temperature. Carbon dioxide was bubbled through the solution to neutralize remaining alkali. An equal quantity of ether was added and the solution was filtered. The salt cake on the funnel was washed with 95 per cent ethanol. The ethanol was removed at a pressure of 10 to 12 mm. of Hg and at a water bath temperature of  $50^\circ$ . The residue was taken up

<sup>1</sup> We wish to express our appreciation to Dr. Herbert Carter for making available to us this information before its publication.

in 100 cc. of water and 200 cc. of ether and acidified with dilute HCl. The ether layer was separated and the water was reextracted with two 50 cc. portions of ether. The ether extracts were combined and were washed with 50 cc. of saturated  $\text{CaCl}_2$  solution, then with a little water, and finally were dried with sodium sulfate. The ether was removed and the residue was then distilled under reduced pressure. A fraction which boiled at  $84-88^\circ$  at 10 to 12 mm. of Hg was collected. This weighed 188 gm. and represented a yield of 84 per cent of the theoretical amount of ethyl  $\alpha$ -bromo- $\beta$ -ethoxypropionate. The pure product was distilled at  $103-104.5^\circ$  at 13 mm. The product was somewhat unstable. When it was allowed to stand at room temperature before distillation or was distilled in larger amounts, a considerable amount of an acrylate was obtained in a low boiling fraction.

188 gm. of the ester were placed in a 1 liter flask with 200 cc. of water and a drop of phenolphthalein solution. 170 cc. of 5 N NaOH were added slowly with shaking under the tap. At the end of the addition the solution was shaken for  $\frac{1}{2}$  hour and then a slight excess of an equivalent quantity of  $\text{H}_2\text{SO}_4$  was added. The solution was extracted with 400 cc. of ether followed by three 200 cc. portions. The ether extracts were washed with saturated sodium sulfate solution and then were dried over sodium sulfate. The ether was distilled and the last traces of ether were removed from the residue *in vacuo*. The oil which remained was added to 2 liters of ammonium hydroxide and the mixture was heated in a steel bomb for 7 hours at  $100^\circ$ .

At the end of the amination the solution was filtered and was distilled *in vacuo* to a syrup. 500 cc. of water were added and the solution was again evaporated to a syrup. The residue was taken up in 800 cc. of 48 per cent HBr and was heated for 3 hours under a reflux. The solution was concentrated to about 500 cc. After the solution was cooled, it was filtered. The filtrate was distilled to dryness at reduced pressure. 500 cc. of water were added and the solution was again distilled to dryness. The residue was taken up in 100 cc. of water and concentrated  $\text{NH}_4\text{OH}$  was added with shaking until the faint odor of ammonia persisted. A liter of absolute ethanol was added slowly and the sides of the flask were scratched. The mixture after being allowed to stand overnight was filtered. The solid was dissolved in 300 cc. of boiling water

and was then treated with norit. After the solution had been filtered and allowed to cool, 41 gm. of colorless crystals were obtained. These were washed with a little cold water and with absolute ethanol. A second crop of crystals was obtained by concentration of the mother liquors and addition of ethanol. After this crop had been recrystallized from dilute ethanol, it weighed 9 gm. The over-all yield of serine was therefore 47 per cent of the theoretical amount.

$C_3H_7O_3N$ . Calculated, N 13.33; found, N 13.3

The *dl*-serine decomposed at 244°. This decomposition point agreed closely with that given by Fischer and Leuchs (5) and likewise agreed with that given by a preparation of serine which we made by the method of Schiltz and Carter. The phenylurea derivative of the *dl*-serine obtained by the present synthesis possessed a melting point of 166–167°. Fischer and Leuchs reported a melting point of 169°. The same derivative prepared from the material synthesized by the Schiltz and Carter method likewise had the same melting point and a mixture of the two compounds showed no lowering. It should be noted that the phenylurea derivative of *dl*-isoserine has a melting point of 183–184° (Neuberg and Mayer (6)).

#### SUMMARY

An economical method of preparing serine from simple materials has been presented. Bromine was added to ethyl acrylate and the ethyl  $\alpha,\beta$ -dibromopropionate was converted to ethyl  $\alpha$ -bromo- $\beta$ -ethoxypropionate by the action of sodium ethylate. This product was then converted to serine by saponification, amination, and hydrolysis. An over-all yield of 47 per cent of the theoretical amount was obtained.

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# STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

## I. TRIOSE PHOSPHATE OXIDATION IN THE PRESENCE OF MALONATE\*

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According to the Szent-Györgyi theory<sup>1</sup> (2-6) the oxidation of intermediary metabolites in animal tissues takes place through the alternate reduction and oxidation of certain 4-carbon dicarboxylic acids which function catalytically as hydrogen carriers and transport the hydrogen of metabolites to the cytochrome system. The metabolite specifically mentioned is triose phosphate ((2) p. 28) and the 4-carbon acids are oxalacetic, malic, fumaric, and succinic. According to the theory, the oxidation of triose phosphate should be represented by the following equations, which may or may not represent direct reactions.

- (1) Triose phosphate + CoI  $\rightarrow$  phosphoglycerate + CoH<sub>2</sub>I
  - (2) CoH<sub>2</sub>I + oxalacetate  $\rightarrow$  CoI + malate
  - (3) Malate + CoI  $\rightarrow$  oxalacetate + CoH<sub>2</sub>I
  - (4) CoH<sub>2</sub>I + fumarate  $\rightarrow$  CoI + succinate
  - (5)\* Succinate + oxidized cytochrome c  $\rightarrow$  fumarate + reduced  
cytochrome c
  - (6) Reduced cytochrome c +  $\frac{1}{2}$ O<sub>2</sub>  $\rightarrow$  oxidized cytochrome c + H<sub>2</sub>O
- Reaction 1 + 2 + 3 + 4 + 5 + 6 = 1 + 4 + 5 + 6 =
- (7) Triose phosphate +  $\frac{1}{2}$ O<sub>2</sub>  $\rightarrow$  phosphoglycerate + H<sub>2</sub>O
- and, Reaction 4 + 5 + 6 =
- (8) CoH<sub>2</sub>I +  $\frac{1}{2}$ O<sub>2</sub>  $\rightarrow$  CoI + H<sub>2</sub>O

\* A preliminary report mentioning this work appeared in Nature (1).

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<sup>1</sup> The many papers bearing on this theory are summed up in Szent-Györgyi's book (2) and review (3).

<sup>2</sup> H + Fe<sup>+++</sup> may be written H<sup>+</sup> + e + Fe<sup>+++</sup>.



One of the strongest arguments in favor of the theory is the fact that small amounts of any of the 4-C acids, when added to a respiring muscle brei, increase consumption of oxygen far beyond the amount required for the complete combustion of the added compound (7-10). The equations above show how this observation is explained by the theory, since according to these reactions the 4-C acids are not used up during the oxidation of triose phosphate. The data have been given an alternate explanation, however, by the work of Krebs and Johnson (11) who showed that the catalytic function of the 4-C acids could be explained on the basis that all are precursors of oxalacetic acid which condenses with a carbohydrate breakdown product, believed to be pyruvic acid, with the ultimate production of citric acid. The latter compound was shown to pass through various intermediate stages with the eventual formation of oxalacetic acid, enabling the "citric acid cycle" to be repeated. This theory obviously also explains the catalytic effect of small amounts of the 4-C acids. Furthermore it also explains how fumarate can nullify the inhibition by malonic acid ((2) p. 21, (8, 10)) an observation that has never been satisfactorily explained by the Szent-Györgyi theory.

The effect of malonic acid as an inhibitor has in fact constituted one of the major pieces of evidence in favor of the Szent-Györgyi theory. Malonic acid is the 3-carbon homologue of succinic acid and although it has a high affinity for succinic dehydrogenase it cannot be dehydrogenated and thus is very effective in blocking the oxidation of succinate to fumarate (12). Since malonate was quite effective in blocking the total respiration of muscle brei (7-10), it was suggested that the oxidation of succinate was a vital link in the transport of hydrogen from tissue metabolites to oxygen. The author decided to test the theory by determining whether or not triose phosphate could be oxidized in a reconstructed enzyme system in which the succinate-fumarate system was eliminated by the use of malonate. If it could be shown that malonate inhibited Reaction 7 as well as Reaction 5 (see above) but had no effect on Reactions 1, 2, 3, 4, or 6, one might conclude that the succinate-fumarate system was indeed a necessary link in the transport of hydrogen from triose phosphate or  $\text{CoH}_2\text{I}$  to oxygen, and the theory of Szent-Györgyi would be confirmed. If, on the other hand, Reaction 5 were inhibited and Reaction 7

unaffected, some mechanism of hydrogen transport other than that shown in Reactions 1 to 6 would be indicated.

#### EXPERIMENTAL

*Enzymes*—Two types of enzyme preparations were used. The triose phosphate dehydrogenase preparations were made according to Green, Needham, and Dewan's method (13) for preparing "mutases." Rabbit skeletal muscle was minced finely and extracted with ice water, the extract was precipitated with acetone, and the precipitate was dried *in vacuo*. The dry powder was homogenized in water with the device of Potter and Elvehjem (14) and the suspension was dialyzed. The denatured proteins were centrifuged down and the clear supernatant was used as a source of zymohexase and triose phosphate dehydrogenase.

The succinoxidase preparations (succinic dehydrogenase plus cytochrome system) were prepared according to Stotz and Hastings (15) by extracting well washed pig heart muscle with alkaline phosphate. It was found to be necessary to use only fresh preparations of this enzyme, since some component of the enzyme system appeared to be quite labile even in the cold (1). In addition to the succinic dehydrogenase and the cytochrome system, the preparation contained small amounts of triose phosphate dehydrogenase and considerable amounts of the coenzyme factor "diaphorase" (16, 17), as was shown by the reduction of methylene blue *in vacuo*, with dihydrocozymase as a hydrogen donator.

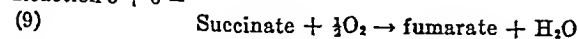
*Coenzyme*—Coenzyme I (diphosphopyridine nucleotide, cozymase) of 95 per cent purity was kindly furnished by Dr. F. Schlenk. It was prepared from ale yeast.

*Apparatus*—Oxygen uptake measurements were made in a conventional Warburg apparatus at 30°.

#### Methods and Results

The effect of malonate on Reaction 5 was studied by observing the effect of the inhibitor on the sum of Reactions 5 and 6, which is simply the oxidation of succinate by molecular oxygen and can be studied in the Warburg apparatus.

Reaction 5 + 6 =



Reaction 9 was studied by adding succinate to the succinoxidase preparation and measuring the oxygen uptake in the presence and

TABLE I

*Effect of Malonate on Oxidation of Triose Phosphate and of Succinate*

Additions	Flask No.									
	1	2	3	4	5	6	7	8	9	10
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
0.5 M phosphate, pH 7.6.....	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Succinoxidase S5a.....	0.20	0.20	0.20	0.20	0.20	0.20	0.40		0.20	0.20
0.1 M sodium succinate*...	0.20	0.20								
0.1 M sodium malonate....		0.20		0.20		0.20				
Triose phosphate Dehydrogenase 1c.....			1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.1% Cozymase 2470.....			0.40	0.40	0.40	0.40	0.40	0.40		0.40
10% hexose diphosphate*..			0.25	0.25	0.25	0.25	0.25	0.25	0.25	
0.4 M sodium fluoride.....					0.15	0.15	0.15	0.15	0.15	0.15
Water.....	2.05	1.85	0.60	0.40	0.45	0.25	0.25	0.65	0.85	0.70

O<sub>2</sub> uptake

	c.mm.	c. mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c. mm.	c. mm.	c. mm.
In 10 min.....	85	0	92	92	83	85	102	0	0	0
" 30 ".....	175	0	178	180	198	201	246	0	0	0

\* These additions were placed in a side arm of the Warburg flasks and added to the main portion after the temperature equilibration had been completed and the stop-cocks closed. The center cups contained 0.3 ml. of 10 per cent KOH with filter paper in all cases although it was probably unnecessary to use alkali here.

absence of malonate. Flasks 1 and 2 in Table I show that the oxidation of succinate was completely inhibited by malonate in a

final concentration of 0.0074 M which was the same as the succinate concentration.

Reaction 7, the oxidation of triose phosphate by molecular oxygen, was studied by measuring the oxygen uptake of a reconstructed enzyme system which was composed of the following additions: hexose diphosphate, triose phosphate dehydrogenase preparation containing zymohexase, cozymase, and the same succinoxidase preparation which was used in succinate experiments; *e.g.*, Flasks 1 and 2, Table I. This complete system oxidized triose phosphate at a rapid rate, as shown with Flask 3. Flask 9 shows that cozymase was needed for the reaction, Flask 10 shows that hexose diphosphate (triose phosphate) was the source of hydrogen, and Flask 8 shows that the succinoxidase preparation was necessary for the oxidation. The next step was to investigate what was being contributed to the mechanism of Reaction 7 (or 8) by this succinoxidase preparation.

Dewan and Green (18), in connection with studies on the enzyme which dehydrogenates  $\text{CoH}_2\text{I}$  (coenzyme factor, diaphorase), have worked with a system which would oxidize triose phosphate and have obtained data very similar to those presented here with Flasks 3, 8, 9, and 10. They concluded that the coenzyme systems could be linked to oxygen via cytochrome oxidase, coenzyme factor, and cytochromes *a* and *b*, which were all present in their heart muscle preparation. They did not consider the possibility that the coenzyme factor might be catalyzing Reaction 4 as Szent-Györgyi suggests (3) and in fact did not report any experiments on the presence or absence of succinic dehydrogenase in their enzyme mixture. In experiments on mechanism it is necessary to define as completely as possible enzyme preparations which are but slightly purified. In the present work the succinoxidase preparation was similar to Dewan and Green's heart muscle preparation and our mixture clearly contains succinic dehydrogenase (Flask 1, Table I). In addition we have shown that the preparation contains considerable amounts of coenzyme factor. We have also demonstrated the presence of cytochrome *c* and cytochrome oxidase according to Stotz, Sidwell, and Hogness (19) and cytochromes *a* and *b* spectroscopically.

It appeared that one could determine whether or not Reaction 8 is to be explained as the sum of Reaction 4 + 5 + 6 quite simply

and directly by adding malonate to a system such as is represented by Flask 3 in Table I. This accordingly was done in the case of Flask 4 and the result clearly shows that the malonate had no effect on Reaction 7 and therefore no effect on Reaction 8. It seems worthy of emphasis that the experiment is strikingly controlled by the results shown with Flasks 1 and 2 in which the same amount of malonate completely blocked Reaction 9 when the same amount of the same enzyme preparation was used. Under the conditions of these experiments, therefore, it appears that Reactions 7 and 8 can proceed by some mechanism other than that proposed by Szent-Györgyi. In a preliminary report (20) we have shown, in contrast to the work of Dewan and Green, that the mechanism includes cytochrome *c*.

Included in Table I are data which show the effect of fluoride with and without malonate on the oxidation of triose phosphate. From the work of Green, Needham, and Dewan it appeared likely that the triose phosphate dehydrogenase preparation contained both  $\alpha$ -phosphoglycerol and lactic dehydrogenases which in the presence of the appropriate oxidized substrate would compete with oxygen for the hydrogen of reduced cozymase; their work indicated that the lactic dehydrogenase was by far the more active. The formation of pyruvate was therefore to be avoided if possible. Pyruvate is believed to be formed subsequent to the breakdown of phosphoglyceric acid to phosphopyruvic acid, a step which is inhibited by fluoride; phosphopyruvic acid is believed to yield pyruvic acid in the presence of the proper enzymes plus adenylic acid and magnesium ions. In the absence of the latter two activators it is doubtful whether the presence or absence of fluoride would have much effect on pyruvate formation. A comparison of Flasks 3 and 4, in which no fluoride was present, with Flasks 5 and 6, to which fluoride was added, shows that the presence of fluoride had very little effect on the initial rate of oxygen uptake but that with time the presence of fluoride actually resulted in a better maintenance of the initial rate. This might be explained by assuming that in the absence of fluoride a small amount of pyruvate was formed by the breakdown of phosphoglyceric acid and in the presence of lactic dehydrogenase this pyruvate was able to compete with oxygen for the hydrogen of reduced cozymase. That this may be the correct explanation

is further supported by the fact that in the absence of fluoride the addition of magnesium ions to the total system in order to stimulate pyruvate formation resulted in a further lowering of oxygen uptake.

#### SUMMARY

1. The effect of malonate on the oxidation of triose phosphate in a reconstructed enzyme system was studied in an attempt to determine whether succinic dehydrogenase is part of the hydrogen transport mechanism between coenzyme I and oxygen, as is postulated by the Szent-Györgyi theory.

2. It was found that the oxidation of triose phosphate was completely unaffected by an amount of malonate which was shown to cause 100 per cent inhibition of the succinic dehydrogenase which was present in the enzyme mixture, suggesting that the oxidation of coenzyme I-linked metabolites can proceed by some mechanism other than that which has been proposed by Szent-Györgyi.

The author takes this opportunity to thank Professor H. von Euler and Dr. Erich Adler for laboratory facilities and helpful suggestions in connection with this problem.

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# THE EFFECT OF ZINC ON ALKALINE PHOSPHATASES\*

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Zinc has been shown to be an element essential to the life and growth of rats, but as yet there is no clear indication of what its function may be in metabolism. There are several reports of the occurrence of zinc in pure biological compounds. Scott and Fisher (1) have shown zinc to be necessary for the crystallization of insulin, although other metals were also effective. Keilin and Mann (2) report that carbonic anhydrase contains 0.32 per cent zinc. However, it has been well established that this enzyme is inhibited by small amounts of added zinc (Meldrum and Roughton (3), and unpublished data from this laboratory). In connection with the rôle of this red blood cell enzyme it may be significant that a zinc-porphyrin compound is excreted in the urine of humans (4). A zinc-porphyrin has also been isolated from diphtherial toxic broth (5). Foster and Waksman (6) have shown zinc to be essential for the growth and fumaric acid production of *Rhizopus nigricans*. Recently Holmberg (7) has found that a purified uricase preparation contains 0.13 per cent zinc.

In previous work from this laboratory (8, 9) we have been able to find no detectable difference in the carbohydrate metabolism of zinc-deficient rats. However, a disturbance of the nitrogen metabolism was observed. This involved a decreased blood non-protein nitrogen, a marked delay in the rate of absorption of amino acids fed by mouth, an increase in fecal nitrogen excretion, a decrease in the daily urinary creatinine excretion, and a marked

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reduction in the pancreatic trypsin content. It was also shown that there is no appreciable decrease in the zinc content of most of the soft tissues of the zinc-deficient animal. However, bone, teeth, and to a lesser degree small intestines do show a decreased concentration of this element. Because of this change our attention was attracted to the possible effect of zinc ions on the activity of phosphatases of the bone and other tissues.

A survey of the literature reveals that Erdtman (10) found a 0.004 mm concentration of zinc ions to produce an 18 per cent inhibition of dialyzed kidney phosphatase. Jenner and Kay (11) state that zinc has no, or slight inhibitory, effect on phosphatases, although they give no experimental details or data. However, Pett and Wynne (12) observed a stimulation of bacterial pyrophosphatases by zinc in small amounts and inhibition at higher levels. Other bacterial phosphatases were inhibited by all concentrations of zinc used.

#### EXPERIMENTAL

Bone phosphatase preparations were made by grinding the cleaned fresh bone (rat femurs), making up to a 5 per cent suspension in water, and allowing to autolyze for 24 hours at 37° in the presence of chloroform. This mixture was then filtered through a Whatman No. 42 paper, producing a water-clear enzyme preparation containing about 4 mg. of dry matter per cc. The crude intestinal enzyme was prepared in the same way except that the washed, whole, rat intestine was minced and made up to a 10 per cent suspension. After the filtration this crude intestinal phosphatase solution had about 14 mg. of dry matter per cc. When this was dialyzed at pH 6.1 for 48 hours, the dry matter content was reduced to 2 mg. per cc. The enzyme solution took on a non-filtrable cloudiness during dialysis which precipitated after a few days in the refrigerator. Toluene was used as a preservative during the dialysis, and a mixture of toluene and chloroform afterwards.

The substrate used in a majority of the experiments to be reported has been sodium  $\beta$ -glycerophosphate, although disodium phenyl phosphate, sodium hexosediphosphate, and sodium pyrophosphate have also been used. These phosphate esters were made up as a 4.8 mm solution (1 cc. = 0.15 mg. of P) in a 50

mm veronal buffer. The final pH was adjusted to 9.2. For each determination 10 cc. of the substrate solution and 0.5 cc. of the enzyme solution in a total volume of 11.5 cc. were incubated at 37°. The reaction was stopped with 2 cc. of a 10 per cent trichloroacetic acid solution, filtered if necessary, and the liberated inorganic phosphorus determined by the customary Fiske-Subbarow method. Blanks were run with every experiment. Any substance added to the reaction mixture was tested for its effect on the substrate alone, under the identical time and temperature conditions of the reaction.

*Effect of Zinc on Phosphatases in Vitro*—In the preliminary experiments on the effect of zinc on the activity of animal alkaline phosphatases *in vitro* the standard King-Armstrong method was used. The substrate for this method is disodium phenyl phosphate, and the incubation time is half an hour at 37.5°. It was found that the activity of crude bone phosphatase was progressively inhibited by increasing concentrations of zinc ion. The effect of zinc on the action of crude kidney phosphatase was also inhibitory, but only to a small degree. However, when crude intestinal phosphatase was tested, zinc was found to have no effect at all.

These preliminary results appeared to indicate a difference in the phosphatases from the three sources, so the work was extended to other substrates. The effect of zinc on the hydrolysis *in vitro* of  $\beta$ -glycerophosphate (21 hours incubation) by the crude bone and kidney phosphatases was more or less markedly inhibitory, thus agreeing with the first results obtained with the phenyl phosphate substrate. However, the effect of zinc on the hydrolysis of glycerophosphate by the crude intestinal phosphatase was markedly activating. Zinc also was found to activate the hydrolysis of sodium hexosediphosphate and sodium pyrophosphate by the crude intestinal phosphatase. These results are shown in Fig. 1. During the last 12 months this stimulating effect of zinc on the hydrolysis of  $\beta$ -glycerophosphate by crude rat intestinal phosphatase has been repeated on approximately 60 to 70 individual preparations, the degree of activation varying between 40 and 110 per cent. Zinc chloride, sulfate, and nitrate are equally effective. The pH of the reaction mixture is not altered by the amounts of zinc salt used in these experiments.

The time course of the hydrolysis of  $\beta$ -glycerophosphate by the crude intestinal phosphatase alone, and with optimum zinc, optimum magnesium, and a combination of optimum amounts of zinc and magnesium, is shown in Fig. 2. From the curves in this chart it can be seen that both of the metal ions undergo an augmentation in their percentage activation effect with time. This has previously been observed for the magnesium ion by Bodansky (13) and Holmberg (14). It should be noticed that the percent-

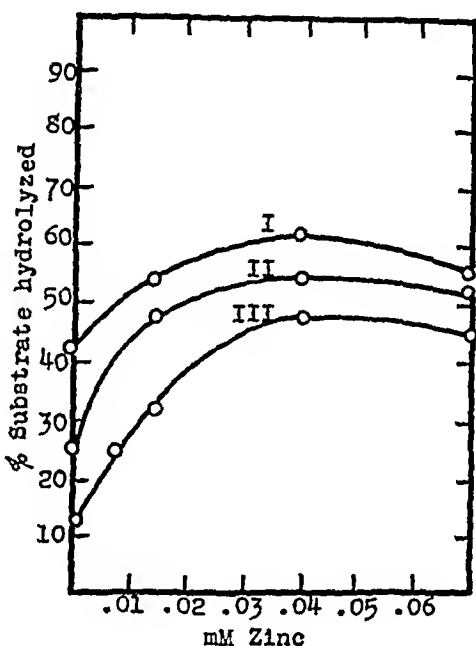


FIG. 1. The effect of zinc on crude intestinal phosphatase activity *in vitro*. 21 hour incubation period at 37°, pH 9.2. Substrates, Curve I, hexosediphosphate; Curve II, glycerophosphate; Curve III, sodium pyrophosphate.

age activation due to zinc at  $\frac{1}{2}$  hour's incubation is slight, amounting to only 5 per cent. This furnishes a possible explanation of the lack of zinc activation of the hydrolysis of the phenyl phosphate substrate by the crude intestinal phosphatase, since here the incubation was only continued for a half hour; and in fact, it has been found that with a longer incubation period zinc does activate the hydrolysis of this substrate. Since the metal ions, zinc and magnesium, together produce an effect greater than

either alone (Fig. 2), it seems possible that the mechanism of activation by these ions is, in part at least, different and independent.

Dialysis of the crude intestinal phosphatase resulted in a complete loss of the ability of zinc ions to activate the enzyme. In fact the intestinal enzyme after dialysis was inhibited by zinc; this observation brings a uniformity into the action of zinc ions on phosphatases from the three sources—bone, intestine, and kid-

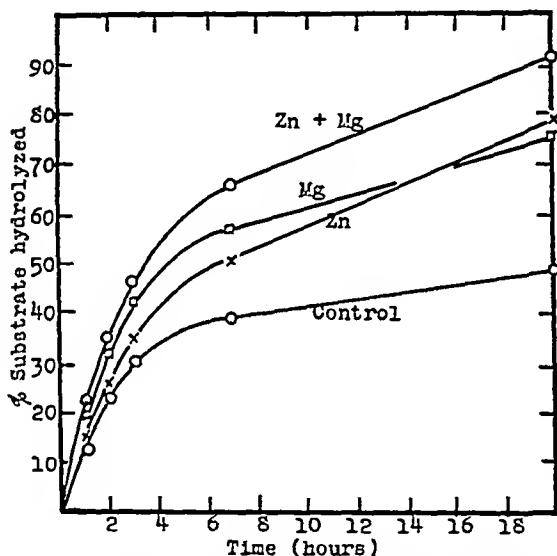


FIG. 2. The time course of the hydrolysis of  $\beta$ -glycerophosphate by crude intestinal phosphatase. Concentration of zinc, 0.03 mM; of magnesium, 0.1 mM.

ney. The degree of the zinc inhibition of intestinal phosphatase increases slowly with the length of dialysis, as is shown in Fig. 3. From the curves shown in Fig. 3, it is evident that the ability of zinc to stimulate the magnesium-activated dialyzing enzyme outlasts the ability of zinc to stimulate the dialyzing enzyme alone. However, this property also is lost when dialysis is sufficiently complete, as is shown in Preparations 2 and 3 of Table I. Also from Table I it is evident that the dialysate, when again added to the dialyzed intestinal enzyme, can restore the zinc

activatability of the enzyme. The dialysate alone, or in the presence of zinc or magnesium or both, had absolutely no effect on the substrate.

These results indicate that there is present in crude intestinal preparations, but seemingly not in bone or kidney preparations, a dialyzable substance which, together with zinc ion, is able to activate phosphatases. This dialyzable zinc coactivator is not destroyed by taking the dialysate to dryness at 100°. It is de-

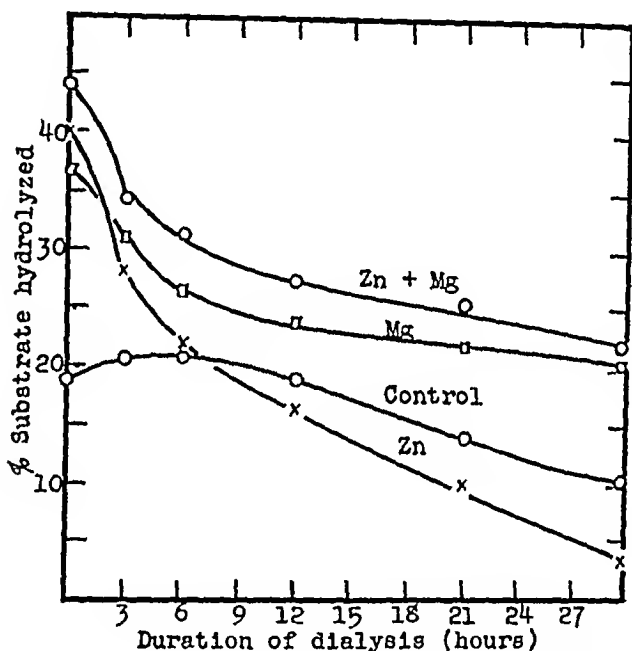


FIG. 3. The change of metal ion action on intestinal phosphatase activity with increasing time of dialysis. Substrate,  $\beta$ -glycerophosphate; incubation period, 21 hours; concentration of zinc, 0.03 mM; of magnesium, 0.2 mM.

stroyed by ashing. It is soluble in water, partially soluble in absolute ethyl alcohol, and completely insoluble in pyridine, acetone, and absolute butyl alcohol. It is soluble in dilute acids and bases, although it seems to be partially destroyed by being taken to dryness in the presence of concentrated ammonia or NaOH. A boiled tissue extract from a fresh intestine has none of this substance. A boiled tissue extract from a portion of the same intestine after a 24 hour autolysis at 37° has high activity.

Since the zinc coactivator appeared to be a product of autolysis,

a casein hydrolysate was tried and found to have a high activity, as is shown in Table II. Following this observation, a group of pure amino acids was tested (the pH of the amino acid solutions having been adjusted before use). As is evident from Table II, all of the  $\alpha$ -amino acids had a more or less strong zine-coactivating property. Their effect seemed to a degree to be inversely related to the length of the chain. The corresponding  $\beta$ -amino acids, keto acids, hydroxy acids, or aliphatic acids had little or no ac-

TABLE I

*Effect of Dialysis for 30 Hours and of Metal Ions on Intestinal Phosphatase Preparations*

Substrate,  $\beta$ -glycerophosphate; incubation period, 21 hours at 37°; final concentration of added metal ions, Zn 0.05 mM, Mg 0.2 mM.

Preparation No.	Metal ion added	Non-dialyzed		Dialyzed		Dialyzed + dialysate	
		P liberated	Change due to metal	P liberated	Change	P liberated	Change
		mg.	per cent	mg.	per cent	mg.	per cent
1	None	0.790		0.587		0.700	
	Mg	0.950	+25	0.644	+10	0.903	+24
	Zn	1.14	+44	0.477	-19	1.04	+48
	Mg + Zn	1.18	+50	0.732	+25	1.06	+49
2	None	0.420		0.249		0.279	
	Mg	0.695	+65	0.464	+86	0.549	+97
	Zn	0.520	+24	0.063	-75	0.448	+61
	Mg + Zn	0.770	+84	0.272	+9	0.804	+187
3	None	0.366		0.216		0.320	
	Mg	0.649	+77	0.403	+88	0.545	+70
	Zn	0.636	+74	0.054	-75	0.470	+47
	Mg + Zn	0.724	+101	0.200	-7	0.611	+92

tivity. This is likewise true of such N compounds as choline and choline. Glutathione had a relatively high activity. This high activity may have been a result of the combined activity of each of the three  $\alpha$ -amino groups in the molecule, indicating that an entirely free  $\text{NH}_2$  group is not necessary, or it may be related in some way to its  $-\text{SH}$  group. It should be noticed, however, that the high percentage activation due to zine in the case of glutathione is due ultimately to the very marked inhibition pro-

duced by the GSH alone and not to a higher final activity brought about by the addition of zinc to the glutathione.

TABLE II

*Action of Amino Acids and Other Organic Compounds\* As Zinc Coactivators for Dialyzed Intestinal Phosphatase*

Substrate,  $\beta$ -glycerophosphate; incubation period, 21 hours at 37°; final concentration of zinc, when added, 0.05 mM.

	Final concentration of organic compound	Hydrolysis of substrate		Activation due to Zn
		No Zn	Plus Zn	
	mg. per cc.	per cent	per cent	per cent
Casein hydrolysate	0	13.6	10.3	
	0.3	13.0	27.0	108
	1.0	11.6	33.5	190
	mM			
Glycine	1.5	10.5	26.1	148
DL-Alanine	1.5	13.0	25.8	100
Aspartate	1.5	12.1	20.8	72
Glutamate	1.5	13.1	19.0	46
Valine	1.5	12.2	20.0	63
Lysine	1.5	11.2	21.0	88
Arginine	1.5	12.0	18.9	57
Cystine	1.0	12.5	24.3	94
GSH	1.5	6.7	21.3	220
$\beta$ -Alanine	1.5	13.7	15.1	9
Choline	1.0	13.7	14.8	8
Cholamine	1.5	13.6	15.2	10
Lactate	1.5	14.8	12.5	
Pyruvate	1.5	13.9	13.7	
Glutarate	1.5	13.7	13.7	
Nicotinate	1.5	13.6	12.7	
Ascorbate	1.5	12.8	12.1	

\* The organic compounds were adjusted to pH 8.5 before use.

From Fig. 4 it is evident that neither zinc nor the amino acid alanine had any effect on the pH optimum of the intestinal phosphatase.

The next point to establish was the optimum concentration of the amino acids in their action as zinc coactivators. The work with the amino acid glycine will be reported in detail. From Table III the following points are apparent. (a) The optimum

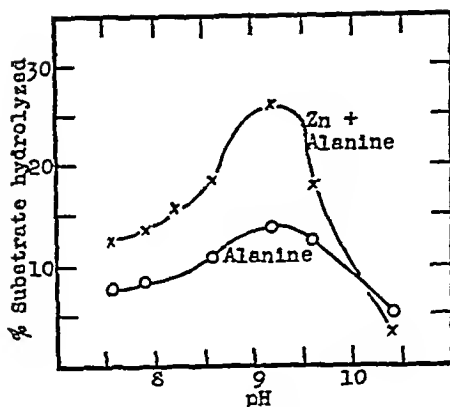


FIG. 4. The pH-activity curve of dialyzed intestinal phosphatase in the presence of alanine (1 mM) and alanine plus zinc (0.03 mM). Substrate,  $\beta$ -glycerophosphate; incubation period, 18 hours.

TABLE III

*Optimum Concentration of Zinc and Glycine for Activation of Dialyzed Intestinal Phosphatase*

Substrate,  $\beta$ -glycerophosphate; pH, 9.2. The figures are the percentage hydrolysis of the substrate.

Time incubated	Zn concentration	Glycine concentration				
		0 mM	0.5 mM	1.0 mM	2.0 mM	4.0 mM
hrs.	mM					
	2					
	0	4.3	4.5	5.0	5.9	3.9
	0.0015	4.8	5.9	7.9	7.7	5.7
	0.006	4.5	6.7	8.5	9.5	9.3
	0.015	3.8	4.8	7.5	10.0	9.2
24	0.035	2.5	3.6	6.7	9.9	10.4
		0 mM	0.9 mM	1.8 mM	4.5 mM	8.7 mM
	0	32.1	25.7	22.0	15.0	12.5
	0.008	38.0	48.0	46.5	38.1	28.0
	0.035	30.9	45.2	52.0	47.1	37.5
	0.075	28.5	36.5	47.0	50.8	47.3
	0.150	26.0	32.4	37.2	45.0	50.0

The bold-faced figures represent the highest values found.



zinc and optimum glycine concentrations are interrelated and depend on each other. A rough proportionality of the concentrations for optimum stimulations is 1 molecule of zinc for every 75 to 100 molecules of glycine; (b) the final optimum reached appears to be about the same for all absolute levels of the zinc-glycine optimum concentrations; (c) the optimum range becomes much broader and flatter when the higher levels of glycine are used. The same general relations apply to the action of zinc and glycine on the  $\beta$ -glycerophosphatase of the kidney (Table IV) and bone (Table V) and the hexosediphosphatase and pyrophosphatase of the dialyzed intestinal preparations (data not shown).

TABLE IV

*Effect of Zinc and of Glycine on Hydrolysis of  $\beta$ -Glycerophosphate by Kidney Phosphatase Preparation*

The figures are the percentage hydrolysis of the substrate in 21 hours.

Zn concentration	Non-dialyzed enzyme	Dialyzed enzyme			
		Glycine concentration			
		0 mm	1.0 mm	3.0 mm	6.0 mm
mm					
0	25.0	13.5	12.8		10.9
0.0015	28.0	16.8	18.7	15.9	13.5
0.005	27.7	10.5	22.0	17.1	16.1
0.015	17.2	8.0		20.0	
0.050	9.5	5.6	14.1	16.3	17.5

As has been shown by Bodansky (13), the degree of activation of phosphatases by the magnesium ion is augmented by the presence of an amino acid. This is also evident from the magnesium curve of Fig. 3. A shifting of the optimum magnesium concentration with the amount of glycine in the reaction mixture is shown in Table VI.

The additions of combinations of zinc and magnesium ions or zinc and manganese ions to dialyzed phosphatases have produced some interesting results. As has been shown in Fig. 3 and Table I, the ability of zinc ion to stimulate intestinal phosphatase activity disappears on dialysis, but the ability of this element to stimulate the same dialyzing phosphatase when it is

activated by an optimum magnesium concentration does not disappear with anything like the same rapidity. With the completely dialyzed enzyme preparation the strong inhibitory

TABLE V

*Action of Zinc and Glycine on Degree of Hydrolysis of  $\beta$ -Glycerophosphate by Bone Phosphatase Preparations at pH 9.2*

The figures are the percentage hydrolysis of the substrate.

Time incubated	Zn concentration	Non-dialyzed enzyme	Dialyzed enzyme			
			Glycine concentration			
			0 mM	1.0 mM	3.0 mM	6.0 mM
hrs.	mM					
2	0	11.3	8.8	9.0	8.5	7.8
	0.0015	14.6	8.3	9.8	8.7	8.8
	0.0045	11.2	6.7	9.6	10.0	9.2
	0.015	6.5	4.5	7.0	9.7	10.0
	0.030	4.4	3.0	5.0	8.7	9.3
21	0		34.0	30.5	25.6	23.0
	0.003		31.7	36.6	34.0	29.8
	0.005		14.5	29.0	34.4	31.0
	0.025		6.4	14.7	26.2	32.8

The bold-faced figures represent the highest values found.

TABLE VI

*Effect of Glycine on Magnesium Activation of Dialyzed Intestinal Enzyme*

Substrate,  $\beta$ -glycerophosphate; incubation period, 18 hours at 37°. The figures are the percentage hydrolysis of the substrate.

Mg concentration	Glycine concentration		
	0 mM	1.0 mM	3.0 mM
mM			
0	19.8	19.8	18.4
0.04	26.6	28.6	28.0
0.10	28.6	31.3	33.6
0.40	28.8	32.8	36.3

effect of zinc and the activating effect of magnesium tend to cancel each other (Table I).

In Figs. 5 and 6, the effect of combinations of zinc and manganese upon crude bone phosphatase is shown. It is apparent

that zinc alone inhibits in the concentrations used, and that manganese alone has a slight activating effect. However, when the two ions are added together at a minimum ratio of Mn:Zn of about 10:1, the activity of the phosphatase is brought to a point higher than by Mn alone. Thus the crude bone enzyme reacts to the two metals in a way analogous to the response observed in the incompletely dialyzed intestinal enzyme. This peculiar relation of the metal ions to phosphatase activity was used to establish the reversibility of the zinc inhibition. The addition

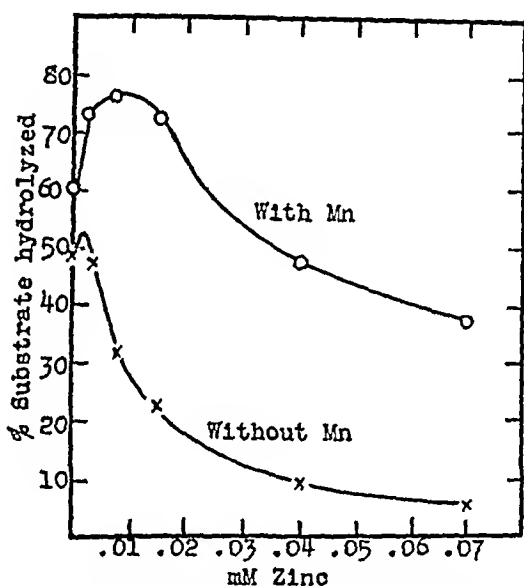


FIG. 5. The effect of increasing amounts of zinc alone, and in the presence of a constant amount of Mn (0.08 mM), on the activity of crude bone phosphatase. Substrate,  $\beta$ -glycerophosphate; incubation period, 21 hours.

of the stimulating ion (Mg or Mn) to the zinc-inhibited reaction mixture after a certain period of incubation resulted in the complete restoration of activity to that attained when the two ions were added from the beginning of the incubation period. These results are shown in Table VII. From this, it is evident that the responses of the incompletely dialyzed intestinal phosphatase and the crude bone enzyme are similar.

*Relation of Zinc to Phosphatases in Vivo*—The phosphatases of the bone and intestines of zinc-deficient animals (prepared as

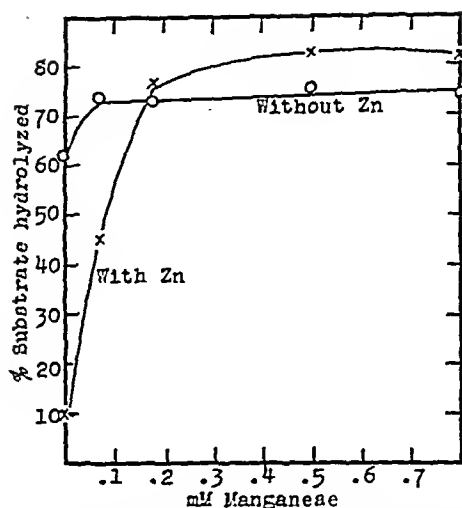


FIG. 6. The effect of increasing amounts of Mn alone, and in the presence of a constant amount of Zn (0.04 mM), on the activity of crude bone phosphatase. Substrate,  $\beta$ -glycerophosphate; incubation period, 21 hours.

TABLE VII

*Reversibility of Zinc Inhibition of Phosphatases*

Substrate,  $\beta$ -glycerophosphate; final concentration of ions, when added, Zn 0.05 mM, Mg 0.2 mM, Mn 0.1 mM; Enzyme A, 24 hour dialyzed intestinal phosphatase, Enzyme B, non-dialyzed bone phosphatase. The figures are the percentage hydrolysis of the substrate.

Enzyme	Metals added	Time incubated			
		0.5 hr.	1.0 hr.	2.0 hrs.	3.0 hrs.
A	None	21.3	33.0	43.1	47.5
	Zn	16.5	22.0	28.5	32.0
			Mg added	50.0	62.1
	Mg	29.5	39.1	54.1	60.2
	Zn + Mg	29.1	43.1	58.2	64.5
		10 hrs.	20 hrs.	40 hrs.	70 hrs.
B	None	38.0	48.8	62.8	70.0
	Zn	6.7	8.7	11.3	14.0
			Mn added	57.0	77.5
	Mn	39.8	51.1	65.0	72.5
	Zn + Mn	43.2	54.0	70.0	78.0

described by Hove, Elvehjem, and Hart (9)) and of rats on a high (toxic) zinc diet have been determined. The crude preparations were used throughout.

*Zinc Deficiency*—From the data in Table VIII it can be seen that the intestinal phosphatase of the zinc-deficient rats is considerably lower than that in normal controls. There was also a slight, but possibly insignificant, lowering of the bone phosphatase.

TABLE VIII

*Intestinal Phosphatase Content of Zinc-Deficient and Control Rats*

Substrate, glycerophosphate; enzyme, crude intestinal preparations; incubation period, 21 hours at 37°.

Group	Per cent hydrolysis of substrate				
		Plus 0.06 mm Zn	Per cent increase in activity due to Zn	Plus 0.2 mm Mg	Per cent increase in activity due to Mg
Zinc-deficient rats	32.1	56.5	75.5		
	18.5	47.0	154	43.8	105
	33.0	61.5	87.3	56.4	51.6
	21.6	43.7	102	45.2	107
	16.4	34.0	108		
	32.8	63.0	92.5		
	37.5	74.0	96.0	57.1	58.0
Average.....	27.4	54.3	102	50.6	80.4
Normal control rats	46.6	85.0	82.5		
	48.0	71.0	48.1	63.0	25.0
	44.5	63.2	41.5	61.2	38.0
	27.5	51.4	94.0	49.5	76.6
	60.0	73.2	22.0	72.0	19.8
Average.....	45.3	68.7	57.6	61.4	39.8

tases. When optimum zinc is added *in vitro* to these crude intestinal preparation reaction mixtures, the response of the preparations from the zinc-deficient rats is much greater than that of preparations from the normal rats. This difference in the reaction to zinc *in vitro* cannot be due to a lower zinc content in the preparations from the deficient rats, since an entirely analogous difference exists with respect to the addition of mag-

nesium ion, *in vitro*, as is shown in Table VIII. The possible explanations are that the crude preparations from the zinc-deficient rats have a lower amino acid content, or that there is a less abundant formation of intestinal phosphatase in the zinc-deficient animal. If the latter is the case, then the greater percentage activation by the metal ions may, perhaps, be related to the greater dilution of these enzyme solutions.

*Rats on High Zinc Diet*—Seven adult male rats were placed upon a stock ration and seven similar rats on the stock ration to which had been added 0.5 per cent of zinc (as zinc sulfate).

TABLE IX

*Effect of Feeding High Zinc Diet (0.5 Per Cent Zinc As Zinc Sulfate) to Normal Adult Male Rats for 4 Weeks*

	7 control rats		7 rats on high zinc diet	
	Range	Average	Range	Average
Bone ash, %.....	59.6 -63.0	61.3	54.8 -59.0	56.4
Zn of dry bone, mg. per gm....	0.20- 0.27	0.23	0.76- 1.25	0.97
Bone phosphatase, % hydrolysis of substrate in 21 hrs....	28.0 -34.0	31.0	46.1 -55.4	50.0
Intestinal phosphatase, % hydrolysis of substrate in 21 hrs.....	13.5 -27.0	21.4	16.7 -41.2	28.7
Intestinal phosphatase after dithizone extraction .....	13.5 -25.0	20.3	12.9 -30.0	21.5
Loss of activity due to dithizone extraction, %.....		5.0		25.8

Bone ash, zinc content of the bones, and bone and intestinal phosphatase were determined (Table IX). In confirmation of the work of Waltner (15) a decreased bone ash was noticed when the high zinc diet was fed. Ionic zinc was removed from the intestinal phosphatase preparations by shaking with dithizone (in  $\text{CCl}_4$ ) followed by pure  $\text{CCl}_4$  to remove any adsorbed dithizone. The activity of the intestinal phosphatases after this treatment was considerably less for the rats on the high zinc diet and only slightly less for the rats on the basal diet. This indicates that the higher level of phosphatase activity in the rats on the high zinc diet was actually due to the higher water-extractable zinc content in their intestinal walls.

## DISCUSSION

The marked activation of crude intestinal phosphatase by minute amounts of zinc ion has been shown to occur only in the presence of a dialyzable, organic substance which is present in the crude intestinal preparations but seemingly present to a much less degree in kidney and bone enzyme preparations. There is no evidence that this compound is in any way related to the co-phosphatase of Albers (16). It has been shown that several pure amino acids have an action equal to that of the zinc coactivator present in the crude intestinal preparation. However, it has not been established that the amino acids liberated by the autolysis of the intestinal mucosa are the only compounds with a zinc-coactivating property present in the crude preparation. In fact, the lability of a portion of the natural coactivator to strong alkali suggests the possible rôle of  $-SH$  groups. The pure amino acids do not show this strong alkali lability.

It has been consistently observed that the crude intestinal enzyme preparation gives no visible precipitate when trichloroacetic acid is added, while the crude bone and kidney enzyme preparations do. This indicates that the hydrolysis of the tissue proteins during the autolysis is more complete in the case of the intestinal preparations and strengthens the assumption that the *natural coactivator is the liberated amino acids*. The explanation for this greater degree of autolysis of the intestinal preparations may be related to the adherence of bits of the pancreas, or pancreatic juice itself, to the intestinal wall, thus furnishing tryptic enzymes to aid the breakdown of proteins. The effect of these tryptic enzymes could not be very great in the autolyzing or stored preparations, since the pH is too low; but during the incubation period of the phosphatase runs these tryptic enzymes can be expected, owing to the now alkaline pH, to be active and produce a certain amount of amino acids from the proteins in solution. Such an amino acid production may be a factor in the marked increase in the percentage activation by zinc and magnesium ions of phosphatase activity with time. It is also possible that the cells of the mucosa may be loaded with amino acids or protein fragments from food digestion.

Both the carboxy group and the  $NH_2$  group in the  $\alpha$  position are essential for zinc coactivation. Yet the wide difference in

the concentration of the zinc and the amino acid for optimum effect makes it difficult to conceive of a final zinc-amino acid complex as being the actual activator. The fact that the zinc ion has a very broad and flat optimum when the amino acid concentration is near that of the substrate concentration suggests a possible rôle of the amino acid as a phosphate transporter or carrier in the dephosphorylation.

Lohmann and Kossel (17) have shown that zinc in a concentration of about  $3 \times 10^{-3}$  mm stimulates the decarboxylation of pyruvic acid by yeast maceration juice plus thiamine pyrophosphate. When they used washed yeast as the source of carboxylase, zinc had only an inhibitory effect. Since in the preparation of the maceration juice the yeast was presumably allowed to autolyze before being ground, it undoubtedly contained a much higher free amino acid content than the washed yeast. It seems a possibility, therefore, that the same relation between zinc and amino acids holds for the activation of this reaction, as we have demonstrated here for the alkaline phosphatases.

The lowered intestinal phosphatase content (or activity) of zinc-deficient rats may be related ultimately to the previously established decrease in pancreatic tryptic activity during the zinc deficiency (9). It had also been shown previously (8) that the zinc deficiency is characterized by a lowered blood non-protein nitrogen and by an increase in the time required for an amino acid given by mouth to pass from the gastrointestinal tract to the blood stream. This delay in the absorption of amino acids may be explained on the basis of intestinal stasis resulting from the decreased pancreatic enzymes, or, if amino acid absorption should be shown to involve phosphorylation the slower amino acid absorption in zinc-deficient rats may be explained on the lowered intestinal phosphatase of these rats. Thus, the *in vitro* relations shown in this paper among zinc, amino acids, and organic phosphoric esters may indicate an *in vivo* relation between these substances and the reactions governing them.

#### SUMMARY

1. Crude intestinal phosphatase activity is increased 40 to 100 per cent by the addition of zinc ions *in vitro*. Crude kidney and bone phosphatase activities are progressively inhibited by concen-



trations of zinc of  $4 \times 10^{-3}$  to  $70 \times 10^{-3}$  mm. This difference in response to zinc cannot be considered as an indication that intestinal phosphatase is different from the others, since after dialysis all three enzymes show a marked inhibition by zinc.

2. The readdition of the dialysate from intestinal phosphatase preparations restores the ability of zinc to activate the dialyzed enzyme.

3. The dialyzable zinc coactivator of intestinal phosphatase preparations is a product of mucosal tissue autolysis. All of the pure  $\alpha$ -amino acids tried have the same coactivating property as the dialysate.  $\beta$ -Amino, keto, hydroxy, or aliphatic acids, or organic amines have little or no activity.

4. The ratio of the concentrations of a pure amino acid (glycine) to zinc ion is about 100:1 for maximum activation. At high glycine concentrations the zinc optimum is broad and flat. At lower glycine concentrations the zinc optimum is sharp.

5. The intestinal phosphatase of zinc-deficient rats is considerably lower than that of normal controls. These results are discussed in relation to previously established disturbances in the nitrogen metabolism in the zinc-deficient rats.

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## PREPARATION AND PROPERTIES OF CRYSTALLINE SALMON PEPSIN\*

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The techniques of crystallization, developed in recent years, have permitted enzyme studies that are far more exact than were possible with the more crude preparations. Among the crystalline enzymes, Northrop's pepsin has been the object of widespread and intensive research (1) which has contributed to the knowledge of enzyme action and mode of operation. In order to further the understanding of pepsin, it seemed worth while to begin comparative studies on pepsins from sources other than beef and swine. In the work to be described the pepsin of the Pacific Coast king salmon (*Oncorhynchus tshawytscha*) was crystallized, and comparative studies have been begun.

Methods for crystallizing swine pepsin have been described by Northrop (2) and Philpot (3). Fortunately, the techniques for handling mammalian pepsin and pepsinogen (4) have been found applicable, with some modification, to fish pepsin.

Most of the fish used as sources of material were caught in traps at La Conner, Washington, and brought under ice to Seattle, where they were dressed, after having been about 18 hours out of water. The stomachs were removed, cleaned, and stored frozen; about 100 were required for each kilo of mucosa. The stomachs from actively feeding fish, caught at sea, were larger and contained more pepsin, but were difficult to ship successfully to Seattle.

\* Taken from a thesis presented by Daniel W. Elam as partial fulfillment of the requirements for the degree of doctor of philosophy, University of Washington, 1940.

Contribution No. 95 from the Oceanographic Laboratories.

*Preparation of Enzyme*—The mucosae were stripped from the muscle, washed and brushed to remove all mucin, and frozen for storage. In a half thawed condition they were finely ground for the extraction. It was found advantageous to extract in alkaline solution, which yielded pepsinogen; acidification of this extract converted the zymogen to pepsin, and at the same time precipitated practically all of the contaminating proteins, which could then be removed by filtration. Denaturation of some of these



FIG. 1. Crystalline salmon pepsin

foreign proteins probably occurred during the alkaline extraction and, to assure this action, more alkali was added than is used by Herriott in the extraction of swine pepsinogen (4). Mucin does not interfere with such a procedure in the extraction of salmon mucosa as it does in the case of beef or swine. The enzyme was salted-out of the clear filtrate by ammonium sulfate, and crystallized by dissolving this precipitate in 0.1 M acetate, pH 5.0, and allowing the solution to stand. The pepsin crystallized as double

refracting needles (Fig. 1). A practical outline of the preparation procedure with some improvements on our previously announced method (5) is given in Table I. The crystals may be stored under half saturated ammonium sulfate in the refrigerator. Solutions of the enzyme are most stable at pH 3.0.

*Evidences of Purity*—All crystalline preparations, even after three successive crystallizations, have shown activities, as measured by Anson and Mirsky's Hb method (6), of 0.23 to 0.26 unit<sup>1</sup> per mg. of protein nitrogen.

That our preparation is homogeneous with respect to protein is indicated by the solubility curves. This criterion for purity has been described by Kunitz and Northrop (7). The amorphous

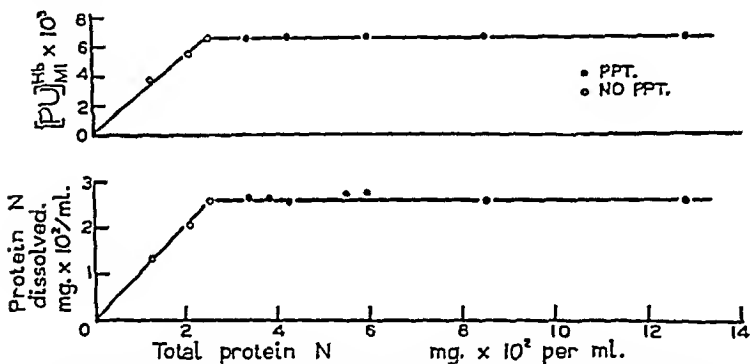


FIG. 2. Solubility of amorphous, twice crystallized salmon pepsin in 0.42 saturated ammonium sulfate; pH 3.0, 25°.  $[PU]$  represents peptic activity of the solution in Hb units per ml.

form of salmon pepsin, precipitated from a fresh solution of fresh and washed crystals, gave the solubility curves of Fig. 2, which are consistent with the demands of the phase rule for one protein species within the limits of our experimental error.

*Composition of Enzyme*—Microchemical analyses of the crystal-

<sup>1</sup> These units are calculated to 35.5°, which is the temperature specified in Anson and Mirsky's definition of the Hb unit. Salmon pepsin is 1.18 times as active at 35.5° as at 25° under the conditions of the determination; this factor for swine pepsin is 1.82 (6). When the two enzymes are compared at 25°, the specific activity of crystalline salmon pepsin is about 1.8 times that of Northrop's pepsin.

line enzyme have shown an elementary composition typical of a protein. The percentage composition is as follows:

C	H	N, Dumas	N, Kjeldahl	S	P	Ash
51.9	6.48	15.62	15.2	1.58	0.031	0.08

The techniques used are described in Pregl (8), with the exception that sulfur was determined by sodium peroxide fusion, a micro modification of a method of the Association of Official Agricultural Chemists (9). Part of the filter cake of crystals used in these analyses was dissolved, and portions of the solution were tested with silver nitrate, barium chloride, and Nessler's reagent. Indications of chloride, sulfate, or ammonium ions were not discernible.

The percentages of tyrosine, tryptophane, and cystine have been determined by the methods of Folin and Marenzi (10). The association of peptic activity with the tyrosine residues is illustrated by the preparations of acetyl (11), nitroso (12), and iodopepsin (13). Our attention was drawn to cystine by the higher sulfur content of salmon pepsin compared to swine pepsin (1), which corresponds in a qualitative way with the higher specific activity of the former as measured by the Hb method. The amino acid percentages are as follows:

	Tyrosine	Tryptophane	Cystine
Crystallized once.....	6.7	1.2	2.0
" twice.....	6.8	1.1	2.1

Salmon pepsin, compared to swine pepsin (14), contains about twice the percentage of cystine, half as much tryptophane, and about seven-tenths as much tyrosine.

The isoelectric point of the crystalline enzyme, prepared by the method described, was estimated by minimum solubility of the amorphous form in ammonium sulfate solution and in alcohol. The pH of least solubility was near 2.0. Crystals, suspended in citrate-phosphate buffer, changed their direction of migration in an electric field between pH 3.0 and 3.1.

### Kinetics

The Hb method (6) was used for all routine assays; the calibration curve for crystalline salmon pepsin is expressed by the

empirical relation, pepsin units =  $0.313 T - 0.0020 T^2 + 0.00068 T^3$ , in which  $T$  represents the milliequivalents of tyrosine in 5 ml. of filtrate, after 10 minutes incubation at  $25^\circ$ . The equation is valid between the limits  $T = 0$  and  $T = 12$ . The proteolytic unit of this equation is based on the liberation of 1 milliequivalent of tyrosine per minute in the standard digest at  $25^\circ$ . In order to obtain  $35.5^\circ$  units, the equation constants should be multiplied by 1.18.

*Influence of pH*—The effect of pH on the initial rate of digestion of 2 per cent Hb is shown in Figs. 3 and 4. In the former case, close correspondence with Northrop's pepsin was secured. These data were obtained with a preparation of salmon pepsin which had been prepared by acid extraction of the mucosa and precipitation with acetone, according to the method of Fenger and Andrew (15). The first precipitate had been dried *in vacuo* over sulfuric acid and stored for 3 years over calcium chloride. At the beginning of this work, the dried preparation was dissolved and fractionated with cold acetone; the fraction soluble in 60 per cent but precipitated by 80 per cent of the organic solvent was retained. Owing to the small quantity of the material, only a few crystals were obtained; they appeared to have the same crystalline form as is illustrated in Fig. 1.

Crystalline salmon pepsin, prepared according to the outline of Table I, exhibited a pH *versus* activity relationship which is plotted in Fig. 4. We have consistently obtained this type of curve with preparations made by this method and, over a short period of time, we have been unable to prepare again by acid extraction a salmon pepsin with the type of function shown in Fig. 3.

The presence of salt markedly increased the rate of digestion. Denatured Hb was found only sparingly soluble in salt concentrations which approach the composition of sea water. However, the rate of digestion as a function of pH and in the presence of 0.1 N NaCl is shown in Fig. 4, Curve B. The same enzyme solution was used for both curves of Fig. 4.

The Hb solutions were prepared by titrating a solution of 5 per cent dialyzed Hb with dilute HCl to the desired pH, and diluting to 2 per cent Hb with distilled water. Sodium chloride was added as a 3.5 N solution. The final pH values were determined by means of a glass electrode, standardized against pH

3.97 phthalate buffer. In all other respects the determinations were performed according to the procedure of the Hb method.

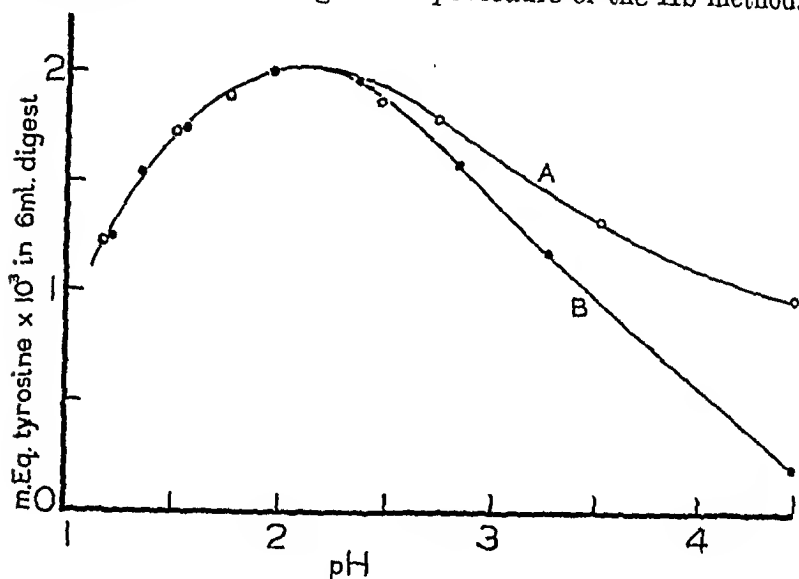


FIG. 3. Dependence of initial rate of digestion of Hb on pH. Curve A, salmon pepsin which had been desiccated; Curve B, Northrop's pepsin, prepared in this laboratory.

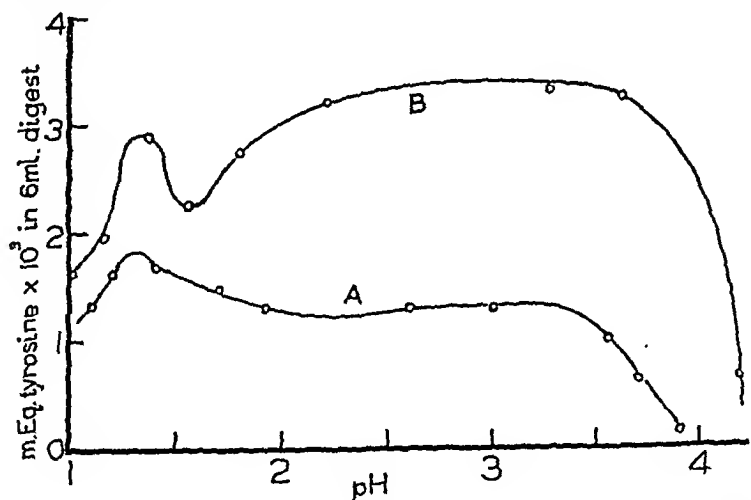


FIG. 4. Dependence of initial rate of digestion of Hb on pH. Curve A, crystalline salmon pepsin in salt-free medium; Curve B, in presence of 0.1 N NaCl.

*Oxidation-Reduction*—In order to ascertain whether oxidation-reduction could account for the two types of activity *versus* pH

TABLE I

*Preparation of Crystalline Salmon Pepsin*

(1) 2500 gm. finely ground mucosa stirred 1 hr. in 7500 cc. water containing 3 moles sodium acetate and 3 moles sodium bicarbonate. Strained through cloth.

Volume 7500 cc.; Hb unit 0.023 per cc., 0.013 per mg. N, total 176;  
% of enzyme 100

(2) To the opalescent pink extract were added slowly 240 cc. 2 N  $\text{H}_2\text{SO}_4$ , bringing the mixture to pH 2.0. To this solution 200 gm. Hyflo super-cel added and solution filtered on a large Buchner funnel whose paper was covered with a thin cake of super-cel. Filter cake washed with 200 cc. 0.001 N  $\text{H}_2\text{SO}_4$ .

Filtrate and washings,

Volume 9690 cc.; Hb unit 0.015 per cc., 0.13 per mg. N, total 158;  
% of enzyme 90

(3) To the clear yellow filtrate from (2) were added 390 gm. ammonium sulfate per liter, bringing the solution to 0.6 saturation; solution stood overnight at 5°. 20 gm. of Hyflo added and solution filtered; filter cake washed with 120 cc. 0.6 saturated ammonium sulfate solution. Filtrate and washings discarded. Filter cake suspended in 300 cc. 0.1 M acetate buffer, pH 4.2, and filtered to remove Hyflo. Filter washed with additional 100 cc. buffer solution.

Filtrate and washings,

Volume 429 cc.; Hb unit 0.34 per cc., 0.14 per mg. N, total 146;  
% of enzyme 83

(4) To the clear filtrate was added N  $\text{H}_2\text{SO}_4$  to produce pH about 2.0; 390 gm. ammonium sulfate per liter added; allowed to stand overnight at 5°. Filtered with suction on hard paper without filter aid. Cracks of draining cake closed with spatula in order to remove as much mother liquor as possible. Filtrate discarded.

(5) Cake from (4) stirred to mush with a small amount of 0.1 M acetate buffer, pH 5.0; more buffer added with stirring until ppt. just dissolved; in all, 175 cc. buffer used. This solution filtered immediately with suction and transferred to a beaker in which it was stirred slowly for 2 or 3 days at room temperature. (It is best to seed with crystals from a former batch.) Crystals filtered off and washed with 50 cc. cold water which had been acidified to pH 4 or 5 with HCl.

Crystal cake,

Hb unit 0.24 per mg. N, total 27; % of enzyme 16

(6) Filtrate and washings from (5) treated by procedure (4).

(7) Recrystallization. Crystal cake from (5) dissolved in 8 cc. 0.1 N  $\text{H}_2\text{SO}_4$ . Solution centrifuged. Supernatant poured into 80 cc. 0.1 M acetate, pH 5.0. Crystallization rapid and complete in 1 hr.

Hb unit 0.23–0.26 per mg. N



curves obtained, the effects of iodoacetic acid and cysteine were investigated. The Hb method was adapted to the experiments. Since iodoacetic acid reacts very slowly with sulfhydryl groups at a pH less than 5.0 (16), the enzyme solution at pH 5.3 was diluted with an equal volume of 0.01 N iodoacetic acid of pH 5.3 and allowed to stand 20 minutes, after which it was mixed with the Hb substrate without further dilution. For comparison, a second aliquot of the same enzyme solution was diluted with an

TABLE II

*Effects of Iodoacetic Acid and Cysteine on Salmon Pepsin*

(1, a) 5 cc. enzyme solution, pH 5.3 (solution containing about 0.0008 Hb unit per cc.) added to 5 cc. 0.01 N iodoacetic acid, pH 5.3; allowed to stand 20 min. 1 cc. of this mixture + 5 cc. 2% Hb digested 10 min. at 25°. M.eq. tyrosine liberated per 6 cc. digest, at pH 1.2, 0.0045; at pH 2.2, 0.0034; at pH 3.4, 0.0038

(1, b) Same as (1, a) except water, pH 5.3, used instead of iodoacetic acid.

M.eq. tyrosine liberated per 6 cc. digest, at pH 1.2, 0.0044; at pH 2.2, 0.0034; at pH 3.4, 0.0034

(2, a) 5 cc. Hb + 0.5 cc. 0.01 M cysteine solution of same pH + 1 cc. enzyme (solution containing about 0.0003 Hb unit per cc.) digested 10 min. at 25°.

M.eq. tyrosine liberated per 6 cc. digest,\* at pH 1.2, 0.0036; at pH 2.2, 0.0028; at pH 3.4, 0.0029

(2, b) Same as (2, a) except that cysteine was added just after addition of trichloroacetic acid.

M.eq. tyrosine liberated per 6 cc. digest,\* at pH 1.2, 0.0036; at pH 2.2, 0.0026; at pH 3.4, 0.0027

\* The color due to cysteine was inhibited by treating the 5 cc. aliquot of filtrate with 1 cc. of 40 per cent formaldehyde and 10 cc. of 0.5 N NaOH, and adding the phenol reagent 5 minutes later (17).

equal volume of water of pH 5.3. The effect of reduction was observed by adding cysteine directly to the Hb solution, as suggested by Anson (17). Results of these experiments comprise Table II. The only influence shown was a slight increase in activity with the addition of either of these reagents, which, in view of the effect of salt, may be attributed to increases in ion concentrations of the solutions.

Since the desiccated preparation of salmon pepsin gave a pH relationship very similar to that of crystalline swine pepsin, we

attempted to learn whether swine pepsin would show an analogous transition during purification. The dependence of activity on pH in the case of swine pepsin has been reported by Sørensen (18), Okuda (19), Ringer (20), Michaelis and Mendelssohn (21), Northrop (22), and Herriott and Northrop (23). These authors state, in most cases, that commercial or purified pepsin had been used, and in no case was the use of a crude mucosa extract reported.

A simple extract of swine pepsin was made as follows: about 2 sq. inches of mucosa, taken from the stomach of a pig just slaugh-

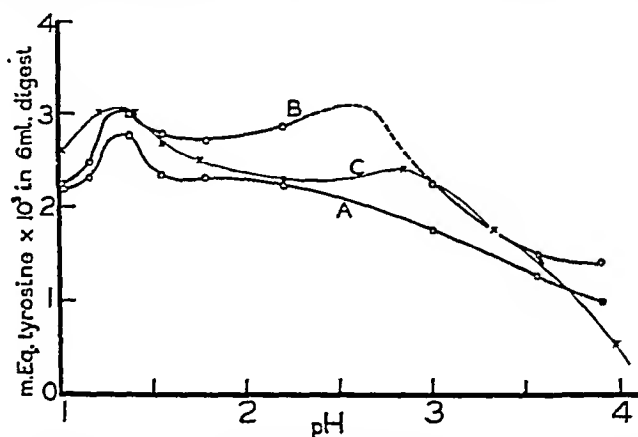


FIG. 5. Dependence of initial rate of digestion of Hb on pH. Curve A, crude extract of fresh hog stomach mucosa; Curve B, in presence of 0.1  $\text{N}$  NaCl; Curve C, extract of fresh stomach mucosa of beef.

tered, was pounded in a mortar with a little sand until thoroughly disintegrated, well mixed with 10 ml. of 0.07  $\text{N}$  HCl, and centrifuged. The cloudy supernatant liquid was diluted to 7 volumes with water and, after standing half an hour, again centrifuged. This clear colorless pepsin solution gave the relationships plotted as Curves A and B in Fig. 5. Curve C of the same figure was obtained with beef pepsin prepared in a similar manner.

The tests with iodoacetic acid and cysteine and the solubility curves lead us to believe that the broad curves of Fig. 4 are not caused by a certain oxidation-reduction state nor by a mixture of enzymes. The curves of Fig. 5 show an analogous situation in

the case of swine pepsin. Whether the occurrence of two forms of dependence of activity on pH is related to the existence of a pepsin-inhibitor complex in the newly formed pepsin, as was demonstrated by Herriott (24), or whether other changes occur in the pepsin molecule, are questions yet to be answered.

*Influence of Temperature*—The ability of cold blooded animals to carry on digestive processes in winter months was pointed out by Hoppe-Seyler (25) and Rakoczy (26) who studied the pepsin

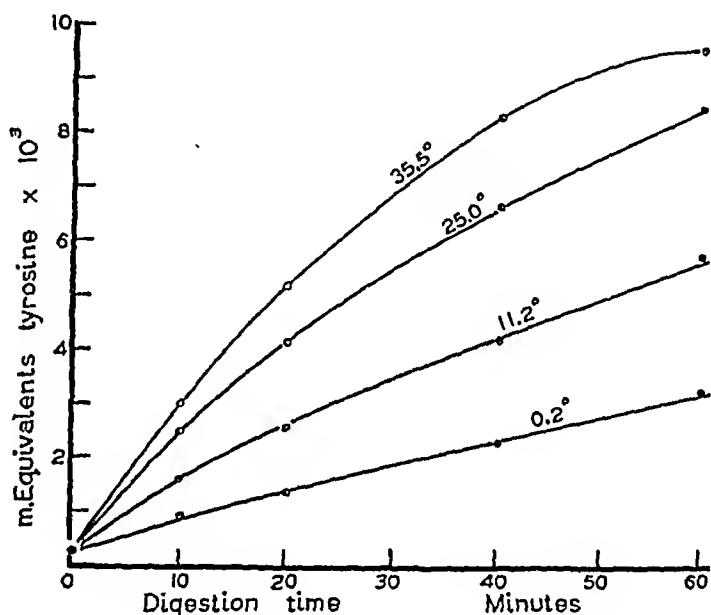


FIG. 6. Digestion of 2 per cent Hb at pH 2.0 by crystalline salmon pepsin at various temperatures. The ordinates represent the tyrosine equivalent liberated in 6 ml. of digest.

of pike. Hykes, Mazanec, and Szecsenyi (27) showed that the perch can digest proteins at almost freezing temperatures. The environment of the salmon living in the North Pacific probably varies between the limits of 5–15°. In Fig. 6 is shown the progress of salmon peptic digestion at pH 2.0 and at various temperatures with 2 per cent Hb as substrate.

We wish to express our thanks to Dr. Ray W. Clough of the National Cannery Association, the Whiz Fish Products Company

of Seattle, and Mr. B. M. Gilmore of the Fishermen's Cooperative Association of Neah Bay, Washington, in appreciation of their aid in securing the salmon stomachs used in this work.

#### SUMMARY

The pepsin of the Pacific Coast king salmon has been crystallized; the method of preparation is described in detail.

An elementary analysis and determinations of tyrosine, tryptophane, and cystine contents have been made. The material is a typical protein, but the needle type crystalline form and the composition show that this enzyme is distinct from Northrop's pepsin.

The enzyme, when prepared by the method described, has a specific activity of about 0.24 Hb unit, and is active over a wide pH range. The presence of salt enhances its activity.

The activity of salmon pepsin is less responsive to changes in temperature than the activity of swine pepsin.

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# LETTERS TO THE EDITORS

## CHOLINE, PANTOTHENIC ACID, AND NICOTINIC ACID AS ESSENTIAL GROWTH FACTORS FOR PNEUMOCOCCUS

Sirs:

A strain of highly virulent Type I pneumococcus has been grown in a medium of pH 7.8 containing, per 10 cc., acid-hydrolyzed gelatin 60 mg., glutamic acid 1 mg., cystine 0.25 mg.,  $\text{KH}_2\text{PO}_4$  50 mg.,  $\text{MgSO}_4$  10 mg., glucose 50 mg., thioglycolic acid 0.5 mg.,

### *Influence of Varying Concentrations of Choline, Pantothenic Acid, and Nicotinic Acid\**

Meat infusion broth controls, Type I, 2.4; Type II, 2.3; Type V, 2.5; Type VIII, 2.4.

Nicotinic acid	Choline	Nephelometer readings					
		Type I		Type II		Type V	Type VIII
		Pantothenic acid, $\gamma$ per cc.					
		1	0.25	1	0.25	1*	1*
$\gamma$ per cc.	$\gamma$ per cc.						
50	10	2.9	>4.7	2.4	>4.7	3.8	3.8
50	5	2.5	2.8	2.4	>4.7	3.5	3.2
50	2.5	2.4	2.5	2.4	2.5	3.4	2.8
50	1	3.4	3.4	3.3	3.6	4.7	3.0
50	0.5	4.2	4.5	4.0	4.6	>4.7	>4.7
10	10	3.3	>4.7	2.5	>4.7	>4.7	4.4
10	5	2.7	3.0	2.3	>4.7	3.5	4.3
10	2.5	2.7	3.7	2.4	2.5	3.4	3.4
10	1	3.7	3.9	3.5	3.6	>4.7	>4.7
10	0.5	4.4	>4.7	4.1	4.7	>4.7	>4.7
2	10	3.7	>4.7	2.5	>4.7	>4.7	>4.7
2	5	2.7	3.3	2.4	>4.7	3.5	>4.7
2	2.5	3.0	3.9	2.4	2.6	3.6	3.5
2	1	3.8	4.2	3.6	3.9	>4.7	>4.7
2	0.5	4.7	>4.7	4.5	4.7	>4.7	>4.7

\* 0.25 microgram per cc. of pantothenic acid gave readings of >4.7 with Types V and VIII throughout.

and flavin 0.001 mg. To this were added choline, pantothenic acid, and nicotinic acid in suitable proportions.

One strain each of highly virulent Types II, V, and VIII pneumococci was grown in a similar medium in which the gelatin hydrolysate was replaced by a mixture of glutamic acid 10 mg., glycine 2.5 mg., asparagine 2.0 mg., leucine 1.5 mg., arginine 0.75 mg., alanine, lysine, cystine, and methionine each 0.5 mg., histidine,  $\beta$ -alanine, and tryptophane each 0.25 mg., norleucine 0.15 mg., oxyproline 0.1 mg., and phenylalanine 0.1 mg.

After test cultures in the various media had been incubated at 37° for 15 to 18 hours, their turbidity was measured with a Gates nephelometer.<sup>1</sup> A reading of 4.7 or more indicated essentially no growth; a reading of 3.0 or less, good growth.

The optimum concentration of choline required by each type was found to be about the same. Nicotinic acid showed a wider range but was, in part, dependent upon the concentrations of both choline and pantothenic acid. The amount of pantothenic acid necessary for growth was influenced by the concentrations of choline and nicotinic acid as well as by the type tested.

The omission of choline or nicotinic acid prevents growth, as does the omission or hydrolysis of pantothenic acid. The presence of thioglycolic acid (or presumably a similar reducing agent) is also essential for growth.

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Received for publication, April 19, 1940

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<sup>1</sup> Rane, L., and Subbarow, Y., *Proc. Soc. Exp. Biol. and Med.*, 38, 837 (1938).

## ACTION OF 4-AMINO-2-METHYLNAPHTHOL ON THE OXIDATION OF CERTAIN SULFHYDRYL GROUPS

Sirs:

Cysteine and reduced glutathione increase clotting time.<sup>1,2</sup> According to Kühnau and Morgenstern<sup>1</sup> these compounds act directly on thrombin. This enzyme when purified gives a faint but definitely positive nitroprusside test.<sup>1</sup> It is well known that various hydrolytic enzymes are *activated* by —SH compounds which reduce —S—S— linkages in the protein of the enzyme. The *inactivating* action of these compounds on thrombin indicates that this enzyme like insulin is only active when most of the sulfur is in the —S—S— form and this would account for the faint nitroprusside test.

The naphthoquinone group present in all compounds with vitamin K activity can be reversibly oxidized and reduced. It was therefore of interest to determine whether such compounds could catalyze the oxidation of —SH groups. For this purpose 4-amino-2-methylnaphthol HCl (kindly supplied by Dr. O. Kamm) was used. It has a high vitamin K activity. In a concentration of 0.05 mg. per cc. it rapidly catalyzes the oxidation to the disulfide of 4.0 mg. of cysteine or thioglycolic acid dissolved in 2.0 cc. of 0.05 M phosphate buffer, pH 7.8. This catalysis is not inhibited by cyanide. It has, however, little effect on the oxidation of reduced glutathione. It causes the rapid disappearance, presumably by oxidation, of the —SH groups in the nucleoprotein of rat liver as measured by the disappearance of the nitroprusside reaction. Finally, in the same concentration, it causes a 50 per cent inhibition in the action of crystalline papain-HCN (kindly supplied by Dr. A. K. Balls) on milk (1.0 cc. of enzyme containing 1.09 mg. of protein N<sub>2</sub> in 5 cc. of milk and 4 cc. of buffer, pH 6.7).

<sup>1</sup> Kühnau, J., and Morgenstern, V., *Z. physiol. Chem.*, 227, 145 (1934).

<sup>2</sup> Mueller, J. H., and Sommers, S., *Science*, 75, 140 (1932). Sterner, J. H., and Medes, G., *Am. J. Physiol.*, 117, 92 (1936).



This compound, and presumably other compounds with the naphthoquinone groups, can oxidize the —SH groups of certain proteins. The presence of naphthoquinones in the body may thus insure the existence of —S—S— linkages or prevent the reduction of such linkages in thrombin when it is formed from prothrombin. It could do this by being attached to the thrombin molecule or by raising the oxidation-reduction potential of the blood or even indirectly by changing the ratio of —SH to —S—S— in the liver.

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## THE RACEMIZATION OF GLUTAMIC ACID

Sirs:

In order to throw further light upon the presence of *d*(-)-glutamic acid in hydrolysates of normal and cancerous tissues,<sup>1</sup> a pure recrystallized sample of 280 gm. of *l*(+)-glutamic acid hydrochloride was heated to gentle boiling with 3 volumes of concentrated hydrochloric acid (sp. gr. 1.19) for 35.5 hours. An analysis of various crops of crystals obtained from this solution

Crops	Weight	$[\alpha]_D^{25}$ in 9 per cent HCl	Nitrogen	<i>d</i> form	<i>d</i> form calculated
	gm.	degrees	per cent	per cent	gm.
Original	280	+31.67	7.58		
1st	256	+31.17	7.55		
2nd	21.9	-4.26	7.55	56.7	12.42
3rd	0.90	+2.23	7.49	46.4	0.42
4th	0.274	+9.9	7.56	34.3	0.094

yielded the results shown in the accompanying table. There was 99.67 per cent recovery of the glutamic acid hydrochloride used, of which 12.934 gm. or 4.6 per cent had been converted into *d*(-)-glutamic acid hydrochloride.

An experiment carried out for a shorter period of heating showed racemization also but in lesser amount. Therefore, the presence of *d*(-)-glutamic acid in protein hydrolysates may be explained as due to racemization during acid hydrolysis. Complete details will be given in a future paper.

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<sup>1</sup> Kōgl, F., and Erxleben, H., *Z. physiol. Chem.*, 258, 57 (1939). Johnson, J. M., *J. Biol. Chem.*, 132, 781 (1940). Chibnall, A. C., Rees, M. W., Williams, E. F., and Boyland, E., *Nature*, 145, 311 (1940).



## PANTOTHENIC ACID DIPHOSPHATE

Sirs:

Since the isolation of pantothenic acid in pure form,<sup>1,2</sup> and the publication of its structure and synthesis,<sup>2</sup> the problem of its rôle in biochemical reactions has received attention. Many of the water-soluble vitamins take part in enzymic reactions as phosphate esters, and the possibility that pantothenic acid phosphates might be of significance was considered. Pantothenic acid acetate<sup>3</sup> and some other derivatives in which the hydroxyl groups are covered are inactive biologically. If the phosphates should be active, one might conclude that either the test organism contained a phosphatase capable of splitting the ester or that the ester itself took part in cell metabolism.

Since details of the synthesis of pantothenic acid have not yet appeared, our method of obtaining the *dl* acid will be indicated.  $\beta,\beta$ -Dimethyl- $\alpha,\gamma$ -dihydroxybutyric acid was made according to Kuhn and Neustadter<sup>4</sup> and its barium salt was acetylated and converted to the acid chloride with thionyl chloride. Union with  $\beta$ -alanine ethyl ester and selective hydrolysis of the ester linkages were performed as previously described.<sup>5</sup> The resulting pantothenic acid was converted to the barium salt with  $\text{BaCO}_3$  and obtained as a white powder by adding ether to its alcoholic solution. Calculated for  $\text{C}_{18}\text{H}_{32}\text{O}_{10}\text{N}_2\text{Ba}$ , Ba 23.9; found, 23.9. 650 mg. of pantothenic acid in 10 cc. of ice-cold pyridine were treated with 1 gm. of  $\text{POCl}_3$ . After 1 hour, the mixture was concentrated to dryness under reduced pressure, and taken up in cold water. The solution was diluted and treated with 20 gm. of

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<sup>2</sup> Williams, R. J., and Major, R. T., *Science*, **91**, 246 (1940).

<sup>3</sup> Woolley, D. W., Waisman, H. A., Mickelsen, O., and Elvehjem, C. A., *J. Biol. Chem.*, **125**, 715 (1938).

<sup>4</sup> Kuhn, M., and Neustadter, V., *Monatsh. Chem.*, **39**, 293 (1918).

<sup>5</sup> Woolley, D. W., Waisman, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, **129**, 673 (1939).

norit A. The norit was washed with alcohol and an aqueous solution of the eluate was neutralized with  $\text{Ba}(\text{OH})_2$ . The barium salt was obtained as a fine, white powder by adding alcohol to its aqueous solution. Yield, 700 mg. Calculated for  $\text{C}_{18}\text{H}_{28}\text{O}_{22}\text{N}_2\text{P}_4\text{Ba}_5$ , P 8.6, Ba 47.8; found, P 8.5, Ba 47.7. Attempts to prepare a crystalline brucine salt were unsuccessful.

Substance	Maximum effect with
<i>dl</i> -Barium pantothenate	1 $\gamma$ per cc.
Ba salt of <i>dl</i> -pantothenic acid diphosphate	Inactive at 70 $\gamma$ per cc.
Reaction mixture	Equivalent to 5 $\gamma$ per cc.

The data summarized in the table show that the diphosphate, in common with other esters of pantothenic acid, is biologically inactive. Assays were performed by the bacterial method.<sup>6</sup> The fact that the crude phosphorylated reaction mixture had some activity probably indicates incomplete phosphorylation. However, possible activity of one of the monophosphates or a pyrophosphate which may have been present in the reaction mixture may explain the result. These compounds are under investigation.

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<sup>6</sup> Snell, E. E., Strong, F. M., and Peterson, W. H., *J. Am. Chem. Soc.*, 60, 2825 (1938).

## A PHOSPHORYLATED OXIDATION PRODUCT OF PYRUVIC ACID

Sirs:

It has been shown that pyruvic acid oxidation is dependent on the presence of inorganic phosphate,<sup>1</sup> but so far it had not been possible to demonstrate the formation of a phosphorylated intermediate. Since pyruvic acid was found to promote adenylic acid phosphorylation,<sup>2</sup> any such intermediate must contain an energy-rich phosphate bond. Phosphopyruvic acid had been excluded by earlier experiments, but recent evidence suggested that acetyl phosphate might be the intermediate.<sup>3</sup> It was found that the phosphate of synthetic acetyl phosphate could be transferred to adenylic acid by an enzyme present in *Bacterium delbrückii*. Tentatively the oxidation was formulated as follows:

$$\text{CH}_3\cdot\text{CO}\cdot\text{COOH} + \text{H}_3\text{PO}_4 + \text{O}_2 = \text{CH}_3\cdot\text{COOPO}_3\text{H}_2 + \text{CO}_2 + \text{H}_2\text{O}_2.$$

In order to obtain a method for determining acetyl phosphate, a closer study of its stability was undertaken. It developed that at room temperature acetyl phosphate is rapidly broken down above pH 8.5 or below pH 2, but is fairly stable between pH 5 and 7. Hence, the magnesia mixture used for the determination of acid-unstable creatine phosphate is too alkaline for this purpose. It has now been found, however, that inorganic phosphate is completely precipitated at *neutral* reaction with calcium chloride in 30 per cent ethyl alcohol, whereas the calcium salt of acetyl phosphate is soluble under these conditions. Moreover, cold trichloroacetic acid can be used for deproteinization, causing prac-

<sup>1</sup> Lipmann, F., *Enzymologia*, 4, 65 (1937). Banga, I., Ochoa, S., and Peters, R. A., *Biochem. J.*, 33, 1980 (1939).

<sup>2</sup> Lipmann, F., *Nature*, 143, 281 (1939). Colowick, S. P., Welch, M. S., and Cori, C. F., *J. Biol. Chem.*, 133, 641 (1940).

<sup>3</sup> Lipmann, F., *Nature*, 144, 381 (1939); in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, 7, 248 (1939).

tically no decomposition. Thus the calcium precipitation method can be used to determine acetyl phosphate by difference.

Pyru- vate	Fluo- ride	O <sub>2</sub> con- sumed	Ca ppt., inorganic P	Direct estimation, inorganic + acid- unstable P	Mg ppt., inorganic + alkali- unstable P	Acetyl phosphate P		Acetyl P Excess O <sub>2</sub>
			(I)	(II)	(III)	(II - I)		
		<i>c.mm.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>c.mm.</i>	
—	—	116	1.30	1.32				
+	—	796	0.57	1.30		0.72	486	0.7
—	+	114	1.29	1.31	1.32			
+	+	734	0.52	1.32	1.31	0.77	556	0.9

Experiments were carried out with enzyme solutions obtained from *Bacterium delbrückii*. After pyruvic acid oxidation for about 1 hour, the cooled solution was deproteinized with 2 per cent trichloroacetic acid, the filtrate rapidly neutralized, and the true inorganic phosphate precipitated with  $\text{CaCl}_2$ . As shown by the data presented in the table, large amounts of inorganic phosphate disappear either in the absence or presence of fluoride, and are nearly equivalent to the extra oxygen consumed, in accordance with the equation, due allowance being made for the imperfect stability of the product. The organic phosphate so formed behaves like acetyl phosphate; *i.e.*, it is split readily and completely by the acid required to determine phosphate directly (II in the table) and (in contrast to creatine phosphate) by alkaline magnesia mixture (III). Colorimetric determination of phosphate was carried out according to Lohmann and Jendrassik.<sup>4</sup>

The demonstration here of a phosphate compound of such limited stability, which is formed metabolically, might well necessitate some revision as to what has usually been regarded as inorganic phosphate in cells and tissues.

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<sup>4</sup> Lohmann, K., and Jendrassik, L., *Biochem. Z.*, **178**, 419 (1926).

## IDENTIFICATION OF THE RICE FACTOR. THE CARBOHYDRATE COMPONENT

Sirs:

In a former paper on the "rice factor"<sup>1</sup> we have presented evidence that glycine is essential for optimum growth of the chick and that chondroitin exerts a growth-promoting effect in certain diets when the glycine and arginine requirements of the chick are met. Further studies of the rôle of chondroitin have revealed that its glucuronic acid component is similarly growth-promoting

A "rice factor" casein basal diet containing 1 per cent of added synthetic glycine was fed.

Supplement	Level, per cent	No. of chicks	Per cent basal gain made
None.....		9	100
Chondroitin.....	3	7	159
Glucuronic acid.....	0.5	9	145
"    "    +.....	0.5	9	150
Galactosamine.....	0.5		
"    .....	0.5	6	75
Hydrolyzed chondroitin.....	3	9	84

On the other hand, the galactosamine component appears to be unessential, if not detrimental. Results which illustrate these statements are given in the table. The basal diets used were similar to those previously described.<sup>1</sup>

Glucuronic acid, alone or with galactosamine, was effective in promoting growth. Galactosamine or acid-hydrolyzed chondroitin containing galactosamine but no glucuronic acid (destruction indicated by a negative Tollens' test) permitted somewhat less growth than that of chicks on the basal diet.

It seems evident that the growth-promoting effect of polished rice and of cartilage in certain experimental chick diets may be a

<sup>1</sup> Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, 134, 213 (1940).



composite result of the presence in these ingredients of glycine, arginine, and glucuronic acid, or a similarly acting substance. Our experiments have clearly differentiated among these separate growth factors.

In addition to increases with glucuronic acid, we have repeatedly obtained growth rate increases with sodium alginate, gum arabic, and certain pentoses. These results indicate that several substances related to glucuronic acid may be active also. It seems possible that certain of these compounds may serve as precursors of the active forms.

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# AN ELECTROLYTIC METHOD FOR THE DETERMINATION OF THE BASIC AMINO ACIDS IN PROTEINS\*

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This work was undertaken in an attempt to fill the need for a simple and accurate procedure for the determination of the basic amino acids in small quantities of protein. Critiques of the existing methods have been made by Mitchell and Hamilton (1), Tristram (2), and Hinsberg and Lang (3). The use of phosphotungstic acid as the preliminary separating reagent for the basic amino acids is subject to many limitations; namely, partial precipitation of other amino acids, variation in solubility of the precipitates in excess of the reagent, and loss of nitrogen on decomposition of the precipitate. This led to the use of the electrolytic isolation of the basic amino acids, originally described by Foster and Schmidt (4).

In outline, the steps of the method to be presented are the following. The protein is hydrolyzed by boiling with 20 per cent hydrochloric acid. An aliquot of the *whole hydrolysate* equivalent to 0.5 to 1.0 gm. of protein is submitted to the electrolytic procedure. From the final catholyte arginine is removed as the flavianate (5), histidine is precipitated with mercuric chloride (6), and lysine is determined by a micro-Kjeldahl analysis of the final fraction.

It is not necessary to remove the humin by filtration, to remove the excess hydrochloric acid by vacuum concentration, nor to remove the amide nitrogen. All of these steps are accomplished

\* This report is from a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

by the electrolytic procedure without the loss of nitrogen usually attendant on adsorptive or mechanical processes. The humin is retained in the middle compartment of the cell. The hydrochloric acid of the hydrolysate is removed by the migration of chloride ions to the anode, where they are discharged as chlorine. The amide nitrogen present as ammonia is volatilized at the cathode from the alkaline solution with the hydrogen there liberated.

### *Details of Method*

*Hydrolysis*—1 to 2 gm. of the protein to be analyzed is hydrolyzed by refluxing for 24 hours with 5 cc. of 20 per cent hydrochloric acid per gm. of protein in an all-glass apparatus. The hydrolysate is diluted to a convenient volume in a volumetric flask, and the total nitrogen is determined by the micro-Kjeldahl procedure. An aliquot representing 0.5 to 1.0 gm. of protein is used for the electrolysis.

*Electrolysis. The Cell*—This (Fig. 1) consists of three wooden sections, the contiguous surfaces of which are covered with gasket rubber. The remaining exposed surfaces are water-proofed with several coats of spar varnish. The capacity of each section is approximately 100 cc. Taps are provided for the rapid and complete removal of solution from the anode and cathode compartments. The assembled cell is held together by long bolts. A piece of quarter inch sheet carbon is used for the anode and platinum gauze for the cathode.

Dissimilar membranes are used at the anode and cathode compartments. The anodic membrane consists of linen coated with gelatin hardened in 4 per cent formaldehyde solution. The cathodic membrane is of parchment paper. This combination is used to minimize endosmotic effects (7-10).

The temperature of the cell is kept at about 20° by circulating tap water through V-shaped glass tubes immersed in the end-chambers. The solution in the center section is agitated by a small air-driven turbine stirrer; the anolyte and catholyte are stirred by the gases liberated there.

The electrical circuit consists of a 100 watt lamp, a 0-1 ampere ammeter, and the electrolytic cell electrodes connected in series to the 110 volt d.c. line. A scale diagram of the entire apparatus is shown in Fig. 1.

*Electrolytic Technique*—The anode and cathode compartments of the assembled cell are filled with distilled water, the middle chamber being only half filled. The current is turned on, and the sample is pipetted into the middle section, which is then filled with distilled water to the same level as the others. The completion of electrolysis is indicated by the current drop (Fig. 2). The contents of the cathode compartment are then run into a

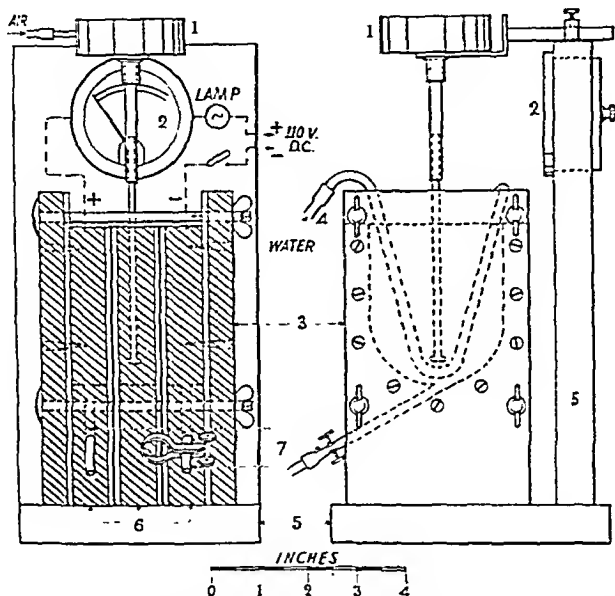


FIG. 1. Drawing of apparatus used for the electrolytic separations. 1 air-driven stirrer, 2 ammeter, 3 electrolytic cell, 4 cooling tube, 5 apparatus stand, 6 compartments of cell, 7 compartment tap.

volumetric cylinder. The chamber is washed several times with distilled water, and the washings added to the original catholyte. The current is turned off. The contents of the anode and middle compartments are discarded. This first electrolysis effects the elimination of hydrochloric acid and the more acidic of the amino acids. The complete separation of the basic amino acids into the cathode chamber is accomplished by a second electrolysis of the primary catholyte.

*First Electrolysis*—Shortly after the sample has been introduced,

the current rises rapidly to 750 to 800 milliamperes, where it remains for about 6 hours, the time depending upon the amount and composition of the protein. About half the contents of the anode chamber is removed at hourly intervals and replaced by distilled water. The electrolysis is complete when the current drops to 100 to 200 milliamperes; the middle compartment solution then gives negative phosphotungstic acid and Sakaguchi tests. The catholyte usually contains about two-thirds of the total nitrogen of the protein used, is alkaline to phenolphthalein, and free of chloride ion. The electrolysis curve (Curve I) for a gelatin hydrolysate is shown in Fig. 2.

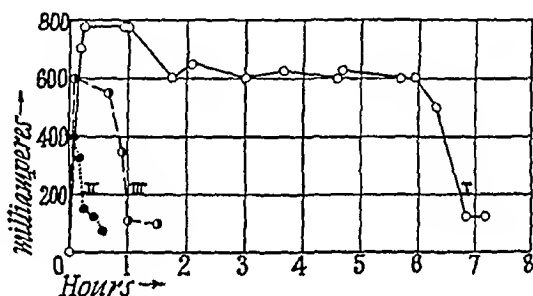


FIG. 2. Electrolysis curves for hydrolyzed gelatin. Total N, 158 mg. Curve I, electrolysis of hydrolysate; Curve II, electrolysis of primary catholyte; Curve III, electrolytic removal of the flavianic acid.

*Second Electrolysis*—A few drops of brom-cresol purple and methyl red are added to the primary catholyte. Dilute  $H_2SO_4$  is then added until the purple color turns to a distinct yellow, pH 5.6 to 5.8. Care must be taken to prevent the appearance of the red color of methyl red, pH 4.6. This solution is introduced into the middle chamber as before. The current rises rapidly to about 450 to 500 milliamperes and then slowly drops to 50 to 75 milliamperes in about 45 minutes, when the electrolysis is complete (Curve II of Fig. 2).

That the basic amino acids had been completely transported to the cathode chamber under these conditions was again shown in preliminary experiments by negative phosphotungstic acid and Sakaguchi tests on the middle compartment.

The middle compartment solution in this electrolysis remains yellow during most of the run, with gradual reddening towards the end. It is not necessary to readjust the pH. An increase in the mean acidity of the amino acids remaining in the center section is to be expected as the basic components leave the chamber. Migration of the neutral amino acids into the cathode chamber is prevented by the alkalinity of the catholyte.

The cathode solution so obtained is strongly alkaline to phenolphthalein and  $\text{Cl}^-$ -free. If it gives a positive test with Nessler's reagent, it is aerated until free of ammonia.

TABLE I

*Data on Primary Electrolysis of Protein Hydrolysates*

Protein hydrolysate	Total N	Basic N transported	Time	Basic N transported per hr.
	mg.	mg.	hrs.	mg.
Edestin.....	149.5	56	4.25	13.1
Horse Hb.....	132	46	6.00	7.65
Fibrin.....	152	42.6	6.00	7.10
Gelatin.....	158.5	37.6	7.00	5.37
Fibrin.....	74	20.7	4.25	4.85
Casein.....	137.5	32	7.00	4.57
Gelatin.....	95.0	23.6	5.50	4.30

*Rate of Electrolysis*—Similar electrolysis curves are obtained with hydrolysates of the various proteins, the only difference being in the duration of the first electrolysis. This appears to be a function of the amount of basic nitrogen in the electrolysate (Table I).

*Recovery Determinations on Electrolytic Procedure*—Electrolyses of arginine hydrochloride were made as described for the protein hydrolysates. 50 cc. of a solution containing 29.0 mg. of N as pure arginine hydrochloride were run into the middle compartment of the cell. The electrolysis was complete in 25 minutes, as indicated by the current rise and drop. The resulting catholyte contained 29.0 mg. of N. In a similar experiment with 5.83 mg. of N as arginine hydrochloride, the catholyte was found to contain 5.82 mg. of N.

*Chemical Procedures for Isolation of Basic Amino Acids*

*Separation and Determination of Arginine*—To the second catholyte, acidified to methyl red with dilute sulfuric acid and heated on the steam bath, is added flavianic acid solution (1.2 moles per mole of arginine expected). After 10 minutes the mixture is allowed to cool to room temperature and stored in the ice box for several days. The arginine flavianate is filtered off, dried, and weighed. A solubility correction of 1.0 mg. of arginine N per 100 cc. of solution<sup>1</sup> is applied to all reported values. The arginine N value calculated from the monoflavianate (11.48 per cent) is reported as per cent of the total N of the protein.

*Electrolytic Removal of Flavianic Acid*—The troublesome removal of the excess flavianic acid from the filtrate from the arginine flavianate by the usual chemical procedures and the consequent loss of nitrogen are obviated by electrolysis. In this process, the arginine flavianate filtrate is introduced into the middle compartment of the cell as previously described. A drop of dilute sulfuric acid is added to both the anode and cathode compartments to increase the initial conductivity. The flavianic acid passes to the anode, histidine and lysine to the cathode. The electrolysis is complete in about an hour. The current change is plotted in Curve III of Fig. 2.

*Histidine Determination*—The method for the preparation of histidine described by Foster and Shemin (6) is adapted to this work in the following manner. The alkaline catholyte from the electrolytic removal of flavianic acid is made just acid to methyl red with dilute HCl. Saturated aqueous solution (6.9 per cent) of  $\text{HgCl}_2$  is added (4 moles per mole of histidine expected). Dilute NaOH is added drop by drop until the red color of methyl red is discharged and a pinkish tinge (pH 6.8 to 7.0) appears with phenol red, when a white flocculent precipitate forms. The resulting mixture (about 100 cc.) is allowed to stand at room temperature for several hours and centrifuged. The precipitate is washed twice with about 10 cc. of water, dissolved in dilute HCl,

<sup>1</sup> This solubility correction was determined for the above conditions of arginine precipitation in the following manner. 100 cc. of the filtrate from the precipitation of pure arginine as the monoflavianate were submitted to electrolysis as described in the next section, and its arginine content estimated by Kjeldahl analysis of the catholyte.

made to volume, and aliquots removed for micro-Kjeldahl determinations. The nitrogen content of the whole solution is estimated as histidine N in per cent of the total N.

*Lysine Determination*—The supernatant and washings of the histidine precipitate are made to volume and aliquots analyzed. The total N of the fraction, less the N due to the solubility of the arginine flavianate, is estimated as lysine N.

### *Analysis of an Artificial Mixture of Amino Acids*

In order to test the precision of the method outlined above, a mixture of amino acids (Table II) was made simulating the composition of a protein hydrolysate. The amino acids used for this experiment were highly purified, with correct nitrogen values. 50 cc. aliquots of this solution were analyzed, equivalent to 223 mg. of total N or approximately 1.4 gm. of protein, and containing 47.8 mg. of basic N. The results are shown in Table III. Attention is directed to the variation of the histidine figures prior to the use of the electrolytic removal of flavianic acid and to their relative constancy after its adoption as a routine step.

### *Analysis of Proteins*

*Gelatin*—Two specimens of gelatin were analyzed. The analysis of Gelatin A (a granular gelatin containing 16.1 per cent N) was made on an aliquot of a 500 gm. hydrolysate. The analyses of Gelatin B (Eastman Kodak gelatin containing 15.16 per cent N) were made on aliquots of a hydrolysate of 10 gm. The results are shown in Table IV.

*Fibrin*—20 gm. of cattle fibrin (Eimer and Amend) were hydrolyzed and made up to 250 cc. A small aliquot was removed for micro-Kjeldahl determination; the protein was found to contain 15.2 per cent N. The analysis is reported in Table IV.

*Casein*—10 gm. of Merck's casein (according to Hammarsten) were hydrolyzed and the hydrolysate was made to 250 cc. Micro-Kjeldahl determinations on this solution showed the protein to contain 13.75 per cent N. The basic amino acid estimations were



TABLE II

*Composition of Amino Acid Mixture*

The mixture contained 15 cc. of HCl and distilled water to make 1 liter.

Amino acid	Amino acid in mixture	Amino acid N in mixture
	gm.	gm.
Glycine.....	10.698	1.995
Proline.....	4.147	0.523
Oxyproline.....	3.577	0.382
Glutamic acid.....	2.518	0.239
Phenylalanine.....	1.091	0.093
Cystine.....	1.226	0.131
Leucine.....	0.420	0.046
Tyrosine.....	1.284	0.100
Histidine HCl·H <sub>2</sub> O.....	1.908	0.387
Arginine HCl.....	1.554	0.413
Lysine HCl.....	0.996	0.153
Totals.....	29.419	4.462

TABLE III

*Results on Analysis of Amino Acid Mixture*

Total N used for each analysis, 223 mg.

N fraction	N present	N recovered						
		Chemical removal of flavianic acid				Electrolytic removal of flavianic acid		
		I	II	III	IV	I	II	Duplicate difference
Primary catholyte, mg..		164	158	160	160	158		
Second catholyte, mg...		46.9	46.8	47.2	46.9	48.0		
Arginine N, mg.....	20.65	20.0	20.3	20.4	20.3	20.4	20.5	
“ “ %.....	9.26					9.15	9.20	0.05
Histidine “ mg.....	19.35	19.50	18.50	18.30	17.10	19.50	19.50	
“ “ %.....	8.68					8.75	8.75	0.00
Lysine N, mg.....	7.6	7.0	7.0	7.2	7.3	7.3	7.4	
“ “ %.....	3.42					3.31	3.37	0.06
Total basic amino acid N, mg.....	47.8	46.7	46.2	46.3	45.5	47.4	47.6	

made on aliquots containing 137.5 mg. of total N. The analytical results are given in Table IV.

*Edestin*<sup>2</sup>—5.746 gm. of edestin were hydrolyzed with 30 cc. of 20 per cent hydrochloric acid for 24 hours. The hydrolysate was diluted to 100 cc. Micro-Kjeldahl determinations indicated 17.30 per cent N in the protein. The basic amino acid analyses reported in Table IV were made on aliquots of the diluted hydrolysate containing 149.5 mg. of total N.

TABLE IV  
*Results of Protein Analysis in Percentages of Total Nitrogen*

Protein	Total N in sample analyzed	Distribution in per cent of total N					
		Arginine		Histidine		Lysine	
			Average		Average		Average
	mg.						
Gelatin A	268	11.5		2.09		6.87	
" B	158.5	11.60		1.14		10.1	
	158.5	11.65		1.14		10.1	
	95.0	11.60	11.62	1.15	1.14	10.05	10.08
Cattle fibrin	152.0	14.0		3.70		11.50	
	152.0	13.95		3.72		11.40	
	74.0	14.0	13.98	Lost	3.71	Lost	11.45
Casein	137.5	7.31		5.32		10.20	
	137.5	7.40	7.36	5.40	5.36	10.23	10.22
Edestin	149.5	26.30		4.25		3.21	
	149.5	26.30	26.30	4.27	4.26	3.23	3.22
Horse Hb	132	6.96		14.50		11.25	
	110	6.95	6.96	14.45	14.48	11.35	11.30

*Horse Hemoglobin*<sup>3</sup>—A sample of horse hemoglobin, three times recrystallized, dialyzed free from chloride, and coagulated by heat, was analyzed. 1.973 gm. of the protein were hydrolyzed as before. The hydrolysate was made to a volume of 50 cc. Micro-Kjeldahl determinations showed the protein to contain 13.40 per cent N. The results of these analyses are shown in Table IV.

<sup>2</sup> Kindly presented by Dr. H. B. Vickery.

<sup>3</sup> Kindly presented by Dr. E. J. Cohn.

*Identification of Amino Acid Fractions*

Experiments are now presented which show that in the protein analyses just recorded, the arginine, histidine, and lysine fractions are not detectably contaminated.

*Arginine*—The most likely contaminants, besides inorganic matter, of the arginine monoflavianate would seem to be arginine and histidine diflavianates. The carbon, nitrogen, and sulfur contents of these three compounds are too nearly alike to be useful criteria of purity. Nor are their decomposition temperatures satisfactory. The determination of the  $\alpha$ -amino N on the flavianates is handicapped by the fact that relatively large amounts are necessary for suitable volumes of gas. The difference between the amino acid nitrogen of arginine monoflavianate and of the

TABLE V  
*Melting Points and Theoretical Percentage Composition of Arginine and Histidine Flavianates*

	M.p.	S	N	C	Amino N	Amino acid N
	°C.					
Arginine monoflavianate.....	258-260	6.54	17.2	39.2	2.85	11.48
“ diflavianate (11)....	220-228	7.95	13.9	38.8	1.75	6.96
Histidine “ .....	251-252	8.17	13.3	39.5	1.79	5.36

possible contaminants appears to offer the best basis for assaying the arginine fraction (Table V).

A weighed sample of the flavianate is suspended in water and dissolved with a minimal amount of dilute NaOH solution. The volume is made to about 100 cc., and dilute H<sub>2</sub>SO<sub>4</sub> is added to bring the solution to about pH 3, just acid to Congo paper. This solution is transferred to the middle compartment of the cell and the electrolysis carried out as described for the removal of flavianic acid. The electrolysis of 300 mg. of arginine flavianate is accomplished in 25 minutes. The catholyte is run into a volumetric cylinder and aliquots taken for micro-Kjeldahl determinations. The amount of N in the whole catholyte represents the amino acid N in the flavianate. Aliquots of this solution are tested for histidine by the diazo reaction, which is sensitive to 1 part of histidine in 200,000 (12).

The results so obtained are shown in Table VI. None of the flavianates showed the presence of histidine and the amino acid N percentages closely approximated the theoretical figure for arginine flavianate (11.48). The melting points, which are really decomposition temperatures, are also given in Table VI. This evidence establishes the purity of the arginine flavianates.

*Histidine N*—The only other amino acids that are known to be precipitated from very dilute solution by mercuric chloride at the pH of the histidine precipitation (pH 7) are cystine and methionine. These, of course, are excluded by the electrolytic separa-

TABLE VI  
*Analytical Data on Basic Amino Acid Fractions*

Protein hydrolyzate	Arginine flavianate		Histidine fraction of protein		Lysine fraction of protein		N pptd. by phosphotungstic acid (see Fig. 3)
	Amino acid N	M.p., uncorrected	Kjeldahl N	$\alpha$ -Amino N $\times 3$	Kjeldahl N	Amino N	
	per cent	$^{\circ}\text{C}$ .	per cent	per cent	per cent	per cent	per cent
Gelatin (Eastman Kodak)	11.57	259-260	1.14		10.10	10.11	88.7
Cattle fibrin...	*	*	3.42	*	11.50	*	*
Casein.....	11.45	257-259	5.32	5.47	10.20	10.23	89.2
Edestin.....	11.50	260-262	4.27	4.25	3.23	3.20	73.5
Horse Hb.....	11.50	257-258	14.50	14.53	11.25	11.30	86.7

\* Discarded.

tion of the bases. Aliquots of this fraction were tested for ammonia with Nessler's reagent. The possible presence of arginine was tested for by the Sakaguchi reaction, which is positive in the presence of arginine in a dilution of 1:1,000,000 (13). Uniformly negative results were secured. The determination of the amino N in this fraction was made by the method of Van Slyke (14). The values so obtained were multiplied by 3 and calculated for histidine N as per cent of total N in the protein. These figures and the corresponding Kjeldahl nitrogen values are listed in Table VI. The close conformity of the histidine fractions to these criteria of purity makes it evident that the nitrogen so reported is all in the form of histidine.

*Lysine N*—Aliquots of this fraction from the different proteins

were submitted to estimation of the amino nitrogen according to the method of Van Slyke. The reaction was allowed to proceed for 30 minutes. The Sakaguchi and Nessler tests were both consistently negative. Since the precipitation of lysine by phosphotungstic acid (15, 16) is not complete, being influenced by conditions of acidity, reagent concentration, and lysine concentration, experiments were made to determine the completeness of precipitation with an authentic specimen of pure lysine hydrochloride under standard conditions. The conditions adopted were total volume of test 10 cc., lysine N between 0.1 and 0.7 mg., phospho-

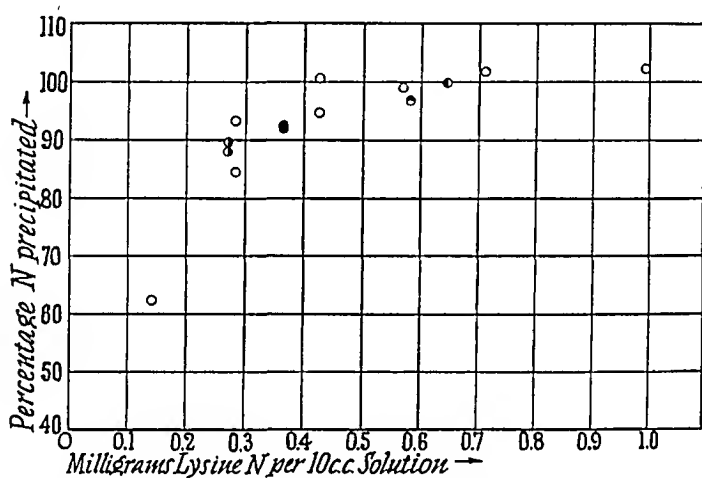


FIG. 3. Comparison of precipitability with phosphotungstic acid of lysine fractions and N of authentic lysine. ○, authentic lysine; ●, casein; ●, edestin; ○, gelatin; ●, horse hemoglobin.

tungstic acid in the ratio of 250 mg. per mg. of lysine N, acidity 5 per cent sulfuric acid, 3 days in the ice box. After centrifugation, the supernatant was analyzed by the Kjeldahl procedure. The results are shown in Fig. 3.

Since the experimental solutions are filtrates from the histidine precipitate and hence contain  $\text{HgCl}_2$ , the effect of the presence of this substance on the precipitation of lysine by phosphotungstic acid was investigated. The presence of 6.9 mg. of  $\text{HgCl}_2$  in 10 cc. of test solution completely prevented the precipitation of 0.426 mg. of lysine N. This indicated the necessity of the removal

of Hg from the solutions prior to the phosphotungstic acid test. This was accomplished by the use of  $\text{H}_2\text{S}$ , the excess of which was removed from the filtrate by a stream of nitrogen. The solutions were treated with phosphotungstic acid under the conditions noted above. The percentage of nitrogen precipitated in each instance is plotted in Fig. 3. The relation of these points to the solubility curve of the known lysine indicates that the nitrogen of the lysine fractions behaves as does the nitrogen of authentic lysine within reasonable limits of error.

The data resulting from these confirmatory tests (Table VI) are interpreted as follows: The agreement of the amino N and Kjeldahl N figures indicates the absence of histidine, arginine, proline, and oxyproline within the limits of error of the methods used. Negative diazo and Sakaguchi tests confirm the absence of histidine and arginine. A negative Nessler test shows that, in the long reaction period required for the estimation of amino N, ammonia did not contribute to the nitrogen so determined. These tests, however, do not exclude the presence of the monoamino acids. If the nitrogen in the fraction is exclusively lysine, then phosphotungstic acid will precipitate all the nitrogen with the exception of that corresponding to the solubility of the lysine salt. Since the nitrogen in the lysine fractions of the different proteins investigated conformed to these tests, it is concluded that it is lysine nitrogen.<sup>4</sup>

#### DISCUSSION

In a comparison of the method presented with existing procedures two features will be discussed, ease of operation and precision. As previously shown, the electrolytic method is free of all chemical or mechanical operations which make for loss of nitrogen. The first electrolysis, which is the most time-consuming step, requires but little attention. Only one filtration, that of arginine flavianate, and one centrifugation, that of the histidine- $\text{HgCl}_2$  complex, are necessary. The transfers of liquid are kept at a minimum by the use of graduated vessels.

The accuracy of the method is established from the analysis of the amino acid mixture. The precision determined by difference be-

<sup>4</sup> In this work no attempt has been made to differentiate between lysine and hydroxylysine (17).

**TABLE VII**  
*Comparison of Errors of Various Methods for Basic Amino Acid Analysis  
of Proteins*

N fraction	Maximum difference between duplicates in terms of per cent of total N in protein				Average difference between duplicates			
	Electro- lytic	Van Slyke	Tris- tram- Block	Vickery and cowork- ers	Electro- lytic	Van Slyke	Tris- tram- Block	Vickery and cowork- ers
Arginine.....	0.09	1.27	0.65	0.9	0.04	0.73	0.52	0.7
Histidine.....	0.08	0.93	0.20	0.5	0.04	0.79	0.14	0.47
Lysine.....	0.10	1.23	0.24	0.5	0.05	0.61	0.15	0.32

**TABLE VIII**  
*Comparison of Results of Protein Analysis by Electrolytic Method and  
Other Methods*

Protein	Method	Distribution in per cent of total N		
		Arginine	Histidine	Lysine
Gelatin	Electrolytic	11.62	1.14	10.08
	Van Slyke (15)	14.70	4.48	6.32
	" (18)	15.7	6.7	9.8
	" (19)	13.6	0.3	12.1
	Arginase (20)	15.68		
	Flavianic acid (5)	16.45		
Cattle fibrin	Diffavianate (11)	15.3		
	Electrolytic	13.98	3.71	11.45
	Van Slyke (15)	13.2-14.3	3.9	10.8-12.0
	" (21)	10.91	4.36	12.05
	Arginase (20)	14.31		
	Silver (22)	13.97	3.83	10.95
Casein	Diffavianate (11)	14.8		
	Electrolytic	7.35	5.36	10.22
	Van Slyke (23)	7.40	6.2	10.3
	Arginase (20)	7.95		
	Silver (2)	8.4	3.3	7.85
	" (24)	7.7- 8.2	3.2	7.73
Edestin	Diffavianate (11)	7.7		
	Electrolytic	26.30	4.26	3.22
	Van Slyke (15)	26.4-27.7	4.6-6.8	3.8
	Arginase (20)	26.7		
	Silver (2)	26.85	3.0	2.59
	" (24)	23.8-27.4	2.6-3.0	2.2
Horse Hb	Diffavianate (11)	28.9		
	Electrolytic	6.96	14.48	11.30
	Van Slyke (25)	7.8	13.0	11.1
	Silver (26)	6.40	12.40	9.30
	Diffavianate (11)	6.92		

tween duplicate estimations is 0.05 for arginine, 0.00 for histidine, and 0.06 for lysine in terms of per cent of total N in the solution. The reproducibility of results in the analyses of proteins approximates these limits. The comparison of the differences in duplicates found by this method and other procedures is given in Table VII, in which the maximal and average differences in the duplicate analyses of each constituent are listed.

The largest reported differences are given by the method of Van Slyke (15). The precision calculations on the Block procedure are made from the excellent critical study of this method by Tristram (2). When the various modifications and corrections worked out by Tristram are applied, this method has a precision second to the electrolytic procedure. The figures on the macromethod of Vickery and coworkers were computed from published data.

The results obtained in the analysis of the various proteins by the electrolytic method are compared in Table VIII with those obtained by other methods. However, direct comparison of results on the analysis of proteins is of questionable validity except where the source of the proteins is identical. Attention is directed to the difference in composition found in two samples of gelatin analyzed by the electrolytic procedure.

#### SUMMARY

A micromethod is presented for the determination of basic amino acids in hydrolysates of 0.5 to 1.0 gm. of protein. The procedure is based on the preliminary separation of these substances from the other products of protein hydrolysis by electrical transport. This is followed by separation of arginine as the flavianate. The flavianic acid in the filtrate from this precipitation is removed by electrolysis. Histidine is precipitated as the mercuric chloride complex. Lysine is estimated from the residual nitrogen. Evidence is presented for the purity of these fractions.

Analyses of some proteins by this method are presented and the results compared with those obtained by other methods.

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# ON THE OCCURRENCE OF IRON-PORPHYRIN COMPOUNDS AND SUCCINIC DEHYDROGENASE IN MARINE ORGANISMS POSSESSING THE COPPER BLOOD PIGMENT HEMOCYANIN

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The process of oxygen utilization in mammalian tissues appears to proceed through a chain of iron-porphyrin compounds composed of hemoglobin, myoglobin, cytochrome oxidase, and the three cytochromes, *a*, *b*, and *c*. Certain marine organisms, however, possess instead of hemoglobin a copper blood pigment, hemocyanin, which functions in a manner similar to the iron blood pigment (*cf.* Redfield (10)). It is of interest, therefore, to inquire whether these organisms are also lacking in those other iron-porphyrin compounds that form part of the respiratory chain in mammalian tissue. We have accordingly examined for iron-porphyrin compounds various tissues of some of those marine forms possessing hemocyanin which are indigenous to the waters surrounding Woods Hole. We have also sought for the presence of succinic dehydrogenase in these same tissues, since this enzyme appears to be intimately bound up with the cytochrome system and the general respiratory cycle of mammalian tissues (12, 13).

## *Methods*

With the exception of cytochrome oxidase, the iron-porphyrin compounds under consideration possess such well defined absorption spectra that their detection in tissues is possible by direct spectroscopic observation. Oxymyoglobin, according to Theorell (14), possesses absorption peaks centered at  $\lambda$  582 and 542 m $\mu$ , while the non-oxygenated form has a relatively weaker band centered at

$\lambda$  555  $m\mu$ . The three cytochromes on the other hand absorb very strongly in the reduced state and but weakly in their oxidized form. Keilin (3) has located the main bands of reduced cytochromes *a*, *b*, and *c* at  $\lambda$  605, 565, and 550  $m\mu$  respectively. We have, therefore, assumed that the observation of absorption bands in tissue whose positions approximate, both in the presence and absence of oxygen, those described here for myoglobin and the three cytochromes constitutes evidence for the presence of these compounds in the tissue under investigation. For the present purpose a hand spectroscope with an attached wave-length scale proved suitable, a carbon arc lamp being used as a light source. Usually the tissue to be examined was placed between two microscope slides held between screw clamps, the thickness of the tissue being varied as suited the concentration of pigment encountered. If no bands were visible, the tissue was ground with sand, and dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) added to insure reduction of the cytochromes, and the observation repeated. The absence of hemoglobin in the preparations greatly facilitates the direct observation of the cytochrome bands in the untreated tissue. In fact the heart tissue of some of these organisms has proved to be the best unworked material known to the authors for the demonstration of the cytochrome spectra. Myoglobin, except in one case, was usually present in such low concentration that the weak absorption of its reduced form did not interfere with the observation of the reduced cytochrome bands.

Succinic dehydrogenase was tested for by the ability of tissue preparations to catalyze the reduction of methylene blue by sodium succinate. The tissue to be investigated was ground with sand and an equal weight of water. The preparation was then centrifuged, the supernatant discarded, and the residue washed twice more in the same manner with water. The washed tissue was then well ground with 2 ml. of 0.1 M  $\text{Na}_2\text{HPO}_4$  solution for each gm. of original wet tissue and allowed to stand for  $\frac{1}{2}$  hour in the ice box. It was then centrifuged briefly to throw down the sand and coarser tissue particles. The turbid supernatant was decanted off and employed for the test, which was run in evacuated Thunberg tubes. Each tube contained 2 ml. of 0.1 M phosphate buffer, pH 7.2, 0.5 ml. of the tissue preparation, and 0.3 ml. of 0.004 per cent methylene blue. The side tube contained 0.2 ml. of 0.2 M sodium succinate or in the case of the control 0.2 ml. of

water. The tests and usually the preparation of the tissue were carried out at room temperatures (average 25°). In the case of the squid, however, it was necessary to prepare the tissue at ice temperatures in order to retard the rapid disintegration the tissues of this organism undergo when handled at room temperatures. The tissues of marine forms in general appear to be so much more susceptible to handling than mammalian tissues that no attempt was made to prepare fumarase-free succinic dehydrogenase by the treatment employed by Lehmann (7). The presence of fumarase in the preparation is without serious consequences for the purpose at hand.

The direct spectroscopic observation of cytochrome oxidase in most tissues is not possible, either because of its low concentration or because of the fusion of its absorption band with that of cytochrome *a* (cf. Keilin and Hartree (5)). The presence of the three cytochromes in tissue is a good indication, though, that cytochrome oxidase is also present. We have, however, chosen to attempt its detection enzymatically by testing the ability of tissue extracts to catalyze the air oxidation of reduced cytochrome *c*. The tissue extracts were the same as those prepared for the succinic dehydrogenase test, since this enzyme and the cytochrome system are found to accompany each other in this preparation. Cytochrome *c* was prepared from beef heart by the method of Keilin and Hartree (4) and reduced previous to the test by dithionite.

### Results

*Limulus polyphemus* (Horseshoe Crab)—The heart and yellow claw or skeletal muscles of this animal showed the absorption spectra of the three reduced cytochromes in their usual positions. The heart muscle was, however, the tissue richest in cytochrome content and it could be shown that the oxidation-reduction potentials of the three cytochrome components in this tissue decreased in the order *a*, *c*, *b* as found by Ball (1) for the cytochromes of mammalian heart. These same muscles were also found to contain succinic dehydrogenase. In the white skeletal muscle, however, neither cytochrome nor succinic dehydrogenase could be detected. Myoglobin does not appear to be a constituent of any of the muscles of this animal.

Two other pigments of a hemochromogen nature have been

observed in this organism. One of these was found in the abundant clot formed when the blue blood of this animal is shed. Spectroscopic observation of the hemocyanin-free clot showed that it contained a pigment with a sharp absorption band centered at  $\lambda$  555 to 560  $m\mu$ . The addition of a reducing agent, dithionite, to the ground clot caused the band to become more intense. The addition of an oxidizing agent, ferricyanide, caused the band to disappear completely and no new band was visible. The original band could be made to return, however, if the preparation was again reduced. The presence or absence of oxygen did not appear to affect the band. Attempts to concentrate this pigment were unsuccessful. It would appear likely that it is contained in the formed elements that are known to be enclosed within the clot. The clot possessed no succinic dehydrogenase activity. It thus appears that in the blood of this animal there occurs not only a copper blood pigment but also a pigment that possesses the properties of an iron-porphyrin compound.

The other hemochromogen-like pigment was found in the glaucous gray to green eggs of the female. Since the intact eggs were too opaque for observation, they were ground with water and centrifuged. The centrifuged material was composed of three fractions, a heavy white fat layer on top, an intermediate bright pink and turbid aqueous fraction, and a dark greenish precipitate. The aqueous fraction when examined with the spectroscope showed an absorption band lying in the region  $\lambda$  620 to 630  $m\mu$ . Removal of oxygen by evacuation caused no change in this band. If, however, dithionite was added, this band disappeared and a new, more intense band appeared located at  $\lambda$  565 to 575  $m\mu$ . This reduced compound is stable for hours in the absence of oxygen but as soon as oxygen is admitted its band immediately disappears and no bands at all are visible. The compound seems to be destroyed, since the further addition of dithionite will not restore the band. If, however, pyridine is now added, a strong absorption band centered at  $\lambda$  555  $m\mu$  appears, indicating that a hemin group is still present. The instability of this pigment frustrated attempts to effect further purification. The bright pink color of the aqueous fraction is not caused by this hemochromogen compound. Another pigment which appears to be a conjugated protein is also present. This pigment shows no marked absorp-

tion bands and is unaffected by dithionite. It is precipitated from solution by the usual methods for protein precipitation. The prosthetic group, however, resisted all attempts to separate it from its protein carrier.

The greenish water-insoluble residue from the eggs shows no sharp absorption bands. It, however, possesses properties that suggest its further investigation may be of interest. Treatment with acid causes an intensification of the green color, while addition of alkali changes its color to yellow and causes the opaque residue to become quite transparent and sticky. If the original residue is extracted with 95 per cent alcohol, a clear orange-colored solution is obtained. Evaporation of the alcohol leaves a residue soluble in water from which an orange pigment may be extracted with chloroform. The water residue from the chloroform extraction is light yellow and fluoresces strongly in a manner characteristic of the flavins. The presence of flavin in this solution is confirmed by the fact that the pigment can be made to yield a lumiflavin derivative, soluble in chloroform.

*Busycon canaliculatum* (Whelk).—This hemocyanin-bearing animal is perhaps the most remarkable with regard to the presence of iron-porphyrin compounds of all those investigated. The radula muscles of this organism are so loaded with a red pigment that this tissue is as highly colored as mammalian skeletal muscle. The marked red color of these muscles in contrast to the bulk of nearly colorless foot muscles has been noted before by Dakin (2), and Mendel and Bradley (9) have established that the pigment responsible is an iron-porphyrin compound by the isolation of hemin crystals. The following properties of this pigment indicate that it is to be classed as myoglobin. The pigment may be readily extracted from the muscles by water. In the presence of oxygen it shows a strong absorption band at  $\lambda$  570 to 580  $m\mu$  and a weaker band at  $\lambda$  540 to 545  $m\mu$ . Removal of oxygen either by evacuation or addition of dithionite causes these bands to disappear and a new weak band appears at  $\lambda$  560  $m\mu$ . Partial purification of the pigment was effected by half saturation of the water extract of the muscles with ammonium sulfate. The precipitate which formed was centrifuged off and discarded. Saturation of the supernatant with ammonium sulfate precipitated the myoglobin. This precipitate was centrifuged off, dis-

solved in water, and dialyzed in the cold until salt-free. The precipitate which formed was removed, leaving a clear solution of the myoglobin. Attempts to crystallize the pigment by the method employed for mammalian myoglobin by Theorell (14) were unsuccessful. Further purification attempts were made difficult by the ease with which the pigment became denatured.

Examination of the radula muscle residue left after the extraction of the myoglobin showed the three cytochromes to be present in low concentration. A test for succinic dehydrogenase was also positive.

The heart of this animal was also found to contain myoglobin. Its concentration in this organ was, however, much lower than in the radula muscles. On the other hand the concentrations of the cytochromes in the heart muscle were much higher. Cytochrome oxidase and succinic dehydrogenase were both found to be present in this tissue. Other tissues were also examined but no appreciable concentration of iron-porphyrin compounds was noted.

*Homarus americanus* (Lobster)—The heart, claw, and skeletal muscle of this animal were examined and found to contain cytochrome, cytochrome oxidase, and succinic dehydrogenase. The concentration of these constituents in the claw and skeletal muscles was, however, extremely low. Their concentration in the heart muscle was equal to that encountered in the other organisms examined in this study. Myoglobin was not detected in any of these tissues.

*Loligo pealii* (Squid)—The tissues of this animal, which is the most active of the group examined, appear to be relatively rich in the cytochromes. We have observed the characteristic absorption spectra of the three cytochromes in the following tissues, heart muscle, head and neck retractors, mantle, muscles surrounding the mouth, sperm, and the nervous tissue behind the eye sometimes designated as the olfactory organs. The main heart muscle shows the most intense cytochrome bands and it is ideal material for the demonstration of the cytochrome spectra, as the cytochrome *b* and *c* bands are sharply differentiated. Myoglobin was not detected in any of the tissues examined. The heart muscle was rich in cytochrome oxidase and succinic dehydrogenase. The preparation of this tissue, however, for tests for these enzymes must be made at low temperatures. The only other tissue as-

sayed for these enzymes was the head and neck retractor muscles, in which low concentrations were encountered.

*Venus mercenaria* (Quahog)—The occurrence of hemocyanin, though reported, in this organism is open to doubt. The body fluid possesses a faint bluish color suggestive of hemocyanin but we have not been able to convince ourselves that this pigment is actually present. Nevertheless we have found the heart and adductor muscles to contain iron-porphyrin compounds. The heart is the only tissue in which we have detected the cytochrome spectra. It also contains an appreciable amount of succinic dehydrogenase. The heart muscle also contains myoglobin which appears to be similar in properties to that found in *Busycon canaliculatum*. The adductor muscles contain a hemochromogen which resembles myoglobin. In the presence of oxygen a strong absorption band at  $\lambda$  575 to 585  $m\mu$  and a weaker one at  $\lambda$  535 to 545  $m\mu$  can be seen. On removal of oxygen or addition of a reducing agent these bands fade out and a clear sharp band at  $\lambda$  560  $m\mu$  appears. There remains a suggestion of a band in the region of  $\lambda$  585  $m\mu$ . The band of the reduced form at  $\lambda$  560  $m\mu$ , however, appears to be much stronger than that given by *Busycon* myoglobin. The presence of succinic dehydrogenase in the adductor muscle is doubtful.

*Molgula manhattensis* (Sea Squirrel)—The ascidians are reported (cf. Webb (17)) to possess a blood pigment in which the metal is vanadium rather than copper or iron. We have therefore included an ascidian, *Molgula manhattensis*, in our observations. This species is so small that a collection of its blood for a chemical test for vanadium is difficult. The ash of the whole animal minus the tunic has, however, given a weak test for this element. Animals from which the tunics were stripped and the intestines removed were examined spectroscopically. A weak band at  $\lambda$  600  $m\mu$  and a broad faint band at  $\lambda$  550 to 560  $m\mu$  were observed. Grinding of the tissue in the presence of air caused these bands to disappear. They could be made to reappear by the addition of a reducing agent. These bands therefore would appear to be due to the three cytochromes in which the *b* and *c* bands overlap. The whole animal was also employed for preparation of succinic dehydrogenase and gave a weak test for this enzyme.

*Arbacia punctulata* (Sea Urchin)—Though this organism does



not contain hemocyanin, we have examined the eggs and sperm of *Arbacia* because a knowledge of their respiratory catalysts is of interest in view of the original finding of Warburg (15) that the fertilization of these eggs causes a marked increase in their oxygen consumption. We have found the sperm to be rich in cytochrome oxidase, the three cytochromes, and succinic dehydrogenase. Cytochrome *a* appears to be present in greater concentration than cytochromes *b* and *c*. The spectroscopic observation of the eggs is made difficult by the presence of echinochrome. However, if the eggs are ground with water, most of the color due to this pigment can then be eliminated by reduction with dithionite. In such a suspension no cytochrome bands can be detected. If the echinochrome is not sufficiently reduced, a band at  $\lambda$  550  $m\mu$  due to this pigment may be confused with cytochrome *c*. Tests for cytochrome oxidase and succinic dehydrogenase in extracts of the eggs were negative. Though the eggs do not appear to contain detectable amounts of the cytochromes, they do contain an appreciable amount of a hemin, as can be shown by the addition of pyridine to a reduced egg suspension. A typical hemochromogen spectrum is then observed in the region  $\lambda$  550 to 560  $m\mu$ . In one experiment performed with the cooperation of Dr. Krah1, fertilized eggs were also examined for cytochrome with negative results. The amount of cytochrome introduced by the sperm is evidently insufficient to raise the concentration in the egg to a level that is detectable by the methods employed by us.

#### DISCUSSION

We have summarized our findings in Table I. The first four animals listed here definitely possess hemocyanin as a blood pigment. They also contain such iron-porphyrin compounds as myoglobin and cytochrome. It would therefore appear that the process of oxygen utilization in these organisms is similar to that in mammals except for the substitution of hemocyanin for hemoglobin. The reason for the utilization of copper instead of iron by these organisms to form their blood pigment can thus not be ascribed to the inability of these animals to utilize iron or to synthesize the porphyrin prosthetic group characteristic of the iron blood pigments. Why an animal like *Busycon* should employ hemocyanin for a blood pigment and yet possess muscles rich in

the pigment myoglobin, which is so akin to hemoglobin, is indeed puzzling. To be sure, when hemoglobin does appear as the blood pigment, it is contained in a special blood cell. Hemocyanin with its greater molecular size does not appear to need such protection in the blood stream. This may be the decisive factor here in the choice between the two blood pigments. It should be recalled, however, that iron-porphyrin blood pigments (erythrocrurins)

TABLE I

*Relative Concentrations of Certain Iron-Porphyrin Compounds and Succinic Dehydrogenase in Various Marine Forms*

Animal	Tissue	Myoglobin	Cytochrome oxidase	Cytochrome a, b, c	Succinic dehydrogenase
<i>Limulus polyphemus</i>	Heart muscle	—		++	++++
	Claw "	—		+	++++
	White "	—		—	+
<i>Busycon canaliculatum</i>	Heart "	+	+	++	+++
	Radula muscles	++++		+	+++
<i>Homarus americanus</i>	Heart muscle	—	++++	++++	+++
	Claw "	—	±	+	±
<i>Loligo pealii</i>	Heart "	—	++++	++++	+++
	Head and neck retractors	—	±	++	±
<i>Venus mercenaria</i>	Heart muscle	+		++	+++
	Adductor muscle	+		—	±
<i>Molgula manhatlensis</i>	Whole animal	—		+	±
<i>Arbacia punctulata</i>	Sperm	—	++++	+++	+++
	Eggs	—	±	—	—

High concentrations of components are indicated by +++++, while failure to detect them by the methods employed is indicated by —. The sign ± indicates that the enzymatic tests employed for detection of this component were so weak that its presence remains doubtful.

whose molecular weights are similar to the hemocyanins are found in the cell-free blood of certain worms.

It will be observed from the data given in Table I that there is a rough parallelism between the occurrence of succinic dehydrogenase and the cytochrome system. This relationship can also be noted in mammalian tissues and is suggestive that the rôle of this enzyme in the respiratory cycle is an important one, as suggested by Szent-Györgyi (13).

The marked difference in the cytochrome content of *Arbacia* sperm and eggs might lead one to postulate that the marked increase in oxygen consumption of eggs on fertilization is due to the introduction into them of the cytochrome system by the sperm. It must be remembered, however, that Warburg (16) and Loeb and Wasteneys (8) have shown that a similar increase in  $O_2$  consumption occurs in eggs artificially fertilized. Runnström (11) and Korr (6) believe that the increase of oxygen consumption on fertilization is due to an activation of a dormant cytochrome system already present in the egg, because cyanide is able to inhibit the respiration of the fertilized egg but not that of the unfertilized egg. Our inability to detect the cytochrome spectra in either fertilized or unfertilized eggs cannot be taken as evidence against this premise, since the concentration of these pigments may have been too low for detection but yet sufficient to play the rôle assigned to them. However, our ability to detect the presence of hemin in these eggs raises the question whether an iron-porphyrin system other than the cytochrome system and which is also cyanide-sensitive may not be the active catalyst in the respiratory cycle of the fertilized eggs.

#### SUMMARY

Iron-porphyrin compounds were found in the tissues of four marine animals, *Limulus polyphemus*, *Busycon canaliculatum*, *Homarus americanus*, and *Loligo pealii*, whose blood pigment is hemocyanin. Cytochrome oxidase and the three cytochromes are present in the heart and some body muscles of all these organisms. Myoglobin is present in high concentration in the radula muscles of *Busycon*. All tissues possessing the cytochrome system were also found to be rich in succinic dehydrogenase. It is therefore concluded that the process of oxygen utilization in these organisms is similar to that in mammals except for the substitution of hemocyanin for hemoglobin. This substitution can therefore not be ascribed to the inability of these animals to utilize iron or to synthesize the porphyrin group characteristic of the iron respiratory pigments.

Two additional hemochromogens have been observed in *Limulus polyphemus*. One is present in the abundant clot obtained from the blood of the animal. Its reduced form possesses an absorption

band centered at  $\lambda$  555 to 560  $m\mu$ . The oxidized form shows no characteristic band. The other hemochromogen is present in the eggs of this animal. Its reduced form shows an absorption band centered at  $\lambda$  570  $m\mu$ , while the oxidized form has a band centered at  $\lambda$  625  $m\mu$ .

The eggs and sperm of *Arbacia punctulata* were also examined. The sperm was found to contain abundant cytochrome oxidase, cytochromes *a*, *b*, and *c*, and succinic dehydrogenase. Tests for these same compounds in the eggs were negative. Upon addition of pyridine and a reducing agent to a ground egg suspension a strong hemochromogen band centered at  $\lambda$  555  $m\mu$  appeared, indicating that a hemin is, however, also a constituent of the eggs.

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# THE ENDOCRINE CONTROL OF LIPID METABOLISM IN THE BIRD

## III. THE EFFECTS OF CRYSTALLINE SEX HORMONES ON THE BLOOD LIPIDS OF THE BIRD\*

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A distinctive feature of the lipid metabolism of the bird is its response to estrogenic activity (1, 2). The experimental stimulation of the ovaries with pregnant mare serum may more than double the lipid content of the blood of the immature bird. A rise in the lipid level also follows the injection of the ovarian hormone, estrogen. The effects of the latter are not confined to the female; a response was also observed in the male bird. Although it required several weeks for pregnant mare serum to produce a rise, increases in the lipid content of the blood were observed as early as 12 hours after the introduction of estrin.

The present study deals with the effects of the following crystalline preparations on the blood lipids of the bird: *estrone*, *estradiol*, *estradiol benzoate*, *ethinyl estradiol*, *stilbestrol*, *progesterone*, *testosterone*, and *desoxycorticosterone acetate*.

### EXPERIMENTAL

Single comb white Leghorn chickens of the Poultry Division stock were used. All birds were immature at the time they received injections. All preparations were administered by the

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intramuscular route. Unless otherwise stated, the total dose of material for each bird was divided into three equal parts and injected at three intervals: (1) between 9.00 and 10.00 a.m., (2) between 4.00 and 5.00 p.m. of the same day, and (3) again about 9.00 a.m. of the following morning. The crystalline compounds were dissolved in sesame oil. Estradiol solutions in two concentrations were used, 0.416 and 2.5 mg. per cc. All other estrogen solutions contained 2.5 mg. per cc. The solutions of progesterone and desoxycorticosterone acetate contained 10 mg. per cc.; the testosterone solution contained 8 mg. per cc. All control birds received 3 cc. of sesame oil, which was divided into three parts and injected at intervals similar to those employed for birds receiving the crystalline preparations.

Blood was removed by heart puncture 48 hours after the first injection was made, and immediately thereafter the bird was sacrificed for examination of the genital tract. The methods employed for lipid analyses have been described elsewhere (3).

### *Results*

Fifteen immature birds were used as controls for the various experiments described below. The values obtained for free and esterified cholesterol, phospholipids, total fatty acids, and total lipids are summarized in Table I.

*Estrone*<sup>1</sup>—Each of the birds recorded in Table II received intramuscularly 5 mg. of estrone per kilo of body weight. A rise in the level of the blood lipids followed the administration of this amount of estrone, but the response varied considerably. In three of the birds, values for total lipids above 1000 mg. per 100 cc. of whole blood were found 48 hours after the first injection. Values close to or above 800 mg. were observed in four birds. In a single bird (No. 83) the blood contained only 527 mg. of total lipids, but even this amount was more than 100 mg. above the average value observed in the control birds (Table I).

Although increases were noted in phospholipids, neutral fat, and cholesterol, equal rises were not obtained in these three lipid constituents. Notable rises were found in the first two constit-

<sup>1</sup> The estrone employed in this investigation was generously furnished by Dr. J. F. Biehn of the Department of Clinical Research, the Abbott Laboratories.

TABLE I  
Summary of Blood Lipids of Control Birds\*

	No. of birds and sex	Weight	Weight of reproductive tract			Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
			Left gonad	Right gonad	Oviduct	Total	Free	Ester				
		gm.	gm.	gm.	gm.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Maximum	8, ♂	1100	2.0	1.64		132	77	65	355	330	487	117
Minimum		820	0.16	0.13		87	50	19	250	225	344	23
Mean		958	0.45	0.37		109	69	40	306	279	415	92
Maximum	7, ♀	940	0.29		0.20	138	93	57	345	316	455	187
Minimum		670	0.18		0.07	90	62	7	227	197	314	48
Mean		795	0.23		0.16	106	78	27	279	253	385	112

\* All birds received a total of 3 cc. of sesame oil administered in three equal parts.

TABLE II  
Effect of Estrone on Blood Lipids

Bird No. and sex	Weight	Injections*		Weight of reproductive tract			Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
		Estrone†	Oil	Left gonad	Right gonad	Oviduct	Total	Free	Ester				
	gm.	mg.	cc.	gm.	gm.	gm.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
72 ♂	980	4.90	1.95	0.26	0.20		206	117	89	1030	480	1236	643
73 ♂	760	3.80	1.53	0.11	0.08		196	106	90	910	530	1106	498
62 ♂	1020	5.10	2.04	0.17	0.13		207	138	69	1133	543	1340	731
71 ♂	830	4.10	1.68	0.78	0.86		150	101	49	614	440	764	283
84 ♀	970	4.80	1.95	0.19		0.96	134	118	16	700	413	834	412
83 ♀	930	4.60	1.85	0.25		0.62	112	64	48	415	297	527	181
79 ♀	920	4.60	1.85	0.22		0.67	133	91	42	713	412	848	408
75 ♀	840	4.20	1.68	0.17		0.61	137	107	30	675	400	812	385

\* The amounts recorded were divided into three equal parts and administered at the intervals stated in the text.

† Corresponds to 5 mg. per kilo of body weight.



TABLE III  
*Effect of Estradiol on Blood Lipids of Domestic Fowl*

Bird No. and sex	Injections*			Weight of reproductive tract				Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids	
	Weight	Estradiol†	Oil	Left gonad		Right gonad	Oviduct	Total	Free	Ester					
				♂	♀										
															gm.
96 ♂	770	3.84	1.56	0.15			0.17		197	97	100	756	433	953	393
97 ♂	860	4.30	1.74	1.10			1.10		152	73	79	571	325	723	295
98 ♂	880	4.40	1.77	1.06			1.01		193	98	95	618	351	811	314
11 ♂	630	3.14	1.26	0.21			0.16		217	125	92	551	284	766	294
99 ♀	560	2.80	1.14		0.19			0.43	189	100	89	576	340	765	283
10 ♀	800	4.00	1.62		0.25			0.62	170	88	82	342		512	
17 ♀	540	2.70	1.08		0.18			0.35	176	95	81	572	304	748	211
18 ♀	560	2.80	1.14		0.17			0.37	163	86	81	490	324	653	217

1.66 mg. estradiol per kilo

Maximum	980			1.30	0.24	1.21	0.48	138	119	80	341	482	479	151
Minimum	770			0.15	0.16	0.13	0.33	91	56	0	240	232	339	30
Mean	912			0.62	0.21	0.53	0.43	112	83	31	285	290	398	85

0.83 mg. estradiol per kilo

Maximum	1050			1.00	0.22	0.92	0.47	115	64	55	482	463	597	214
Minimum	600			0.52	0.15	0.41	0.36	90	46	30	308	229	395	87
Mean	775			0.70	0.17	0.61	0.40	104	59	46	375	301	498	141

\* The amounts recorded were divided into two or three parts and injected at the intervals stated in the text.

† Corresponds to 5 mg. per kilo.

uents listed above, whereas lesser responses were shown by free and ester cholesterol.

*Estradiol*<sup>2</sup>—Estradiol was administered in three amounts: 0.83 mg., 1.66 mg., and 5 mg. per kilo of body weight (Table III). The smallest and largest doses were divided in three parts and

<sup>2</sup> We are greatly indebted to Dr. Erwin Schwenk, Director of the Chemical Research Division of the Schering Corporation, for generous supplies of estradiol, estradiol benzoate, testosterone, progesterone, and desoxycorticosterone acetate.

injected intramuscularly, as above. The 1.66 mg. dose was given in two parts. Blood was removed in all cases 48 hours after the first injection.

No rise in blood lipids was obtained in the eight birds that received 0.83 mg. per kilo (Table III) nor in the eight that were injected with 1.66 mg. per kilo. A definite response, however, followed the administration of 5 mg. per kilo of body weight. The highest value was obtained in Bird 96, in which total lipids rose to 953 mg. per 100 cc. of whole blood. The response was quite similar to the response to estrone, in that the most significant lipid changes occurred in phospholipids and in neutral fat.

*Estradiol Benzoate*<sup>2</sup>—Esterified estradiol had a more pronounced action than the free diol (Table IV). Estradiol and estradiol benzoate were administered in molecular equivalents, 5 and 6.9 mg. per kilo respectively. The maximum value for total lipid observed following the injection of 5 mg. of estradiol was 953 mg., whereas the maximum and minimum responses with molecular equivalents of the benzoate were 2261 and 1418 mg. per 100 cc. of whole blood. All lipid constituents rose with the benzoate, as with the free diol.

*Ethinyl Estradiol*<sup>3</sup>—Each bird received 5 mg. per kilo; its effects on the lipid picture of the blood are recorded in Table IV. The rises produced by this compound are quite striking. In five of the eight injected birds, values for total lipid were above 1000 mg. per 100 cc. of whole blood. The lowest value found for total lipid was 695 mg., as compared with the highest control value of 487 mg. Significant rises occurred in all constituents examined; namely, cholesterol, phospholipids, total fatty acids, and neutral fat.

*Stilbestrol*<sup>4</sup>—This substance was administered in doses of 5 mg. per kilo; its effects are recorded in Table IV. The most marked response of the estrogens studied was obtained with stilbestrol. In four of the birds, values for total lipids were above 2000 mg. 48 hours after the first injection. A rise was obtained in all lipid

<sup>2</sup> Ethinyl estradiol was kindly supplied by Dr. R. C. Mautner of the Ciba Pharmaceutical Products, Inc.

<sup>4</sup> Stilbestrol (4,4'-dihydroxy- $\alpha,\beta$ -diethylstilbine) was kindly furnished by Dr. C. W. Sondern of the Research Laboratories of George A. Breon and Company, Inc.

TABLE IV

*Effect of Estradiol Benzoate, Ethinyl Estradiol, and Stilbestrol on Blood Lipids of Domestic Fowl*

Bird No. and sex	Weight	Injections*		Weight of reproductive tract			Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
		Hormone	Oil	Left gonad	Right gonad	Oviduct	Total	Free	Ester				
Estradiol benzoate (equivalent to 6.9 mg. per kilo)													
	gm.	mg.	cc.	gm.	gm.	gm.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
57 ♂	360	2.48	0.72	0.09	0.08		187	140	47	1670	705	1857	1191
47 ♂	460	3.17	0.93	0.11	0.10		179	133	46	1600	792	1779	1066
58 ♂	390	2.70	0.78	0.10	0.11		168	115	53	1250	647	1418	804
40 ♂	360	2.48	0.72	0.10	0.12		226	100	126	1850	647	2076	1352
50 ♀	360	2.48	0.72	0.15		0.53	202	133	69	1813	785	2015	1268
59 ♀	400	2.76	0.81	0.18		0.85	228	151	77	2033	745	2261	1507
49 ♀	340	2.35	0.69	0.18		0.50	200	114	86	1830	757	2030	1290
67 ♀	330	2.28	0.66	0.15		0.55	190	139	51	1670	702	1860	1190
Ethinyl estradiol (equivalent to 5 mg. per kilo)													
91 ♂	420	2.10	0.84	0.08	0.09		199	111	88	571	580	770	123
92 ♂	950	4.75	1.92	0.27	0.28		211	105	106	767	428	978	403
93 ♂	680	3.46	1.38	0.20	0.23		198	107	91	820	445	1018	456
94 ♂	580	2.90	1.12	0.13	0.14		166	80	86	529	307	695	260
82 ♀	490	2.46	0.99	0.20		0.64	192	129	63	933	899	1125	285
13 ♀	520	2.60	1.05	0.16		0.66	230	109	121	880	451	1110	490
95 ♀	800	4.00	1.62	0.22		1.00	227	155	72	1300	695	1527	781
29 ♀	430	2.15	0.87	0.11		0.60	254	122	132	1055	499	1309	625
Stilbestrol (equivalent to 5 mg. per kilo)													
52 ♂	830	4.15	1.65	0.46	0.46		208	134	74	1590	810	1798	984
61 ♂	700	3.50	1.41	0.19	0.16		176	108	68	1080	845	1256	455
60 ♂	870	4.35	1.74	0.21	0.19		197	131	66	2360	762	2557	1795
69 ♂	840	4.20	1.68	0.19	0.19		216	131	85	1455	465	1671	1077
89 ♀	570	2.86	1.14	0.21		1.04	206	144	62	1952	795	2158	1365
56 ♀	720	3.60	1.44	0.27		1.15	199	148	51	1727	806	1924	1140
64 ♀	620	3.10	1.23	0.23		0.91	212	154	58	1820	1140	2032	1014
42 ♀	630	3.15	1.26	0.25		1.25	220	145	75	2210	680	2430	1692

\* The amounts recorded were divided into three equal parts and administered at the intervals stated in the text.

constituents; here again the most significant changes occurred in phospholipid and neutral fat. The effects produced in phospholipid are indeed striking. Thus the highest phospholipid value shown in the fifteen control birds of Table I was 330 mg., whereas 48 hours after the first injection of stilbestrol Bird 64 contained 1140 mg. of phospholipid per 100 cc. of whole blood.

TABLE V

*Effect of Testosterone, Progesterone, and Desoxycorticosterone Acetate on Blood Lipids of Eight Birds*

	Weight of bird	Weight of reproductive tract				Cholesterol			Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
		Female		Male		Total	Free	Ester				
		Left gonad	Oviduct	Left gonad	Right gonad							
Testosterone (40 mg. per kilo)												
	gm.	gm.	gm.	gm.	gm.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Maximum...	570	0.26	0.23	0.60	0.50	133	76	65	390	364	495	206
Minimum...	500	0.17	0.20	0.12	0.10	90	62	27	273	227	370	43
Mean .....	541	0.21	0.22	0.24	0.21	105	68	37	336	275	440	137
Progesterone (40 mg. per kilo)												
Maximum...	600	0.24	0.25	0.20	0.14	146	76	72	463	327	576	211
Minimum...	440	0.18	0.20	0.10	0.08	97	58	39	224	204	329	19
Mean.....	501	0.20	0.24	0.12	0.11	120	67	53	364	291	484	129
Desoxycorticosterone acetate (60 mg. per kilo)												
Maximum...	410	0.16	0.12	0.15	0.15	155	90	78	506	390	649	156
Minimum	270	0.12	0.09	0.15	0.06	97	62	23	308	323	409	37
Mean...	313	0.14	0.11	0.09	0.09	133	75	58	392	347	526	113

*Testosterone and Progesterone*—The effects of the administration of crystalline preparations of these compounds are summarized in Table V. Despite the fact that amounts as large as 40 mg. per kilo were injected, there occurred no significant changes in the levels of any of the blood lipid constituents.

*Desoxycorticosterone Acetate*—Preliminary experiments, which

are not recorded here, failed to show any rise in the lipid level of cholesterol, total fatty acids, or phospholipids of the blood following the injection of moderate doses of desoxycorticosterone. The results recorded in Table V show the effects of a very large dose; namely, 60 mg. per kilo. Even this amount failed to produce a change in the content of any of the lipid constituents in the blood.

#### DISCUSSION

The observation, reported from these laboratories (2), that estrogens have a pronounced effect on the lipid metabolism of the bird has been amply confirmed by various investigators (4-6). Zondek and Marx (4) obtained increases in the lipid content of the blood after the injection of estrone, estradiol benzoate, and stilbestrol. Their finding that estrone is less effective than stilbestrol or estradiol benzoate is borne out by the results of the present investigation. Landauer *et al.* (5) produced a rise in the lipid level of the blood of chicks with estradiol benzoate, whereas Riddle and Senum (6) have recently reported similar effects with estrone and dihydroestrone (estradiol) in normal and hypophysectomized pigeons. These workers also claimed a slight effect with desoxycorticosterone acetate, but in the present study no rise in the blood lipids was found even after the injection of 60 mg. per kilo of body weight.

The close relation between oviduct growth and blood lipid level in injected immature birds has been previously cited as evidence for the estrogenic nature of the blood lipid response (1, 2). In the present instance, in which all birds were autopsied 48 hours after the first injection, this relation was indeed striking. Even during this short interval oviduct growth had been stimulated appreciably, and oviducts as much as 8 times the average weight of those of the control birds were obtained. The closeness of the relation between the extent of this oviduct growth and the increase in blood lipid levels is clearly illustrated by the coefficient of correlation between oviduct size (expressed as percentage of body weight) and the total fatty acid content of the blood. This coefficient for the forty-seven females used had the highly significant value of  $0.91 \pm 0.03$ .

The variation in response obtained with the estrogens used in

this investigation is worthy of note. No attempt was made to determine the minimum effective dose of the hormones; nevertheless the results obtained show that the extent of their effects is not the same in the bird and in the rat. A positive blood lipid response was obtained by the injection of 5 mg. of estrone per kilo, an amount shown to contain 5000 (7) to 10,000 (8) rat units. The smallest dose of estradiol employed, namely 0.83 mg. per kilo, was also equivalent to 10,000<sup>5</sup> rat units; yet no response in the bird was obtained with twice this amount. 60,000 rat units of estradiol were required to effect a rise in total lipid to an average value of 741 mg. A difference in response is also evident between estradiol and estradiol benzoate. Estradiol benzoate was found more effective than estradiol in the bird. Thus the average value for total lipid observed after the injection of 6.9 mg. per kilo of estradiol benzoate was 1910 mg. per 100 cc. of whole blood; this value was approximately twice that observed after the injection of the molecular equivalent of estradiol. Since birds and mammals differ in their response to estrogens, it follows that an assay based on their effects in mammals can have no meaning for birds. Stilbestrol and ethinyl estradiol responded more like estrone than estradiol, and, although the correspondence may not have been exact, the accuracy of the rat unit method justifies no closer comparison at the present time.

#### SUMMARY

Crystalline preparations of steroid substances and of stilbestrol were investigated for their effectiveness in raising the lipid level of the blood of the chick.

1. The estrogenic substances—estrone, estradiol, estradiol benzoate, ethinyl estradiol, and stilbestrol—increased the concentration of total fatty acids, phospholipids, and cholesterol in the blood.

2. The most striking response was obtained with stilbestrol. Estradiol benzoate gave a more pronounced rise in the lipid con-

<sup>5</sup> Personal communication from Dr. E. Schwenk. Different investigators have estimated the potency of estradiol as being from 2 to 12 times the potency of estrone (7, 9). The same conclusion holds for the lowest estimate made; on the rat unit basis at least twice as much estradiol as estrone was necessary to produce a response in birds.

stituents of the blood than either its free alcohol or ethinyl estradiol.

3. Testosterone, progesterone, and desoxycorticosterone acetate, even when administered in doses as high as 60 mg. per kilo, had no influence on the lipid level of the blood.

4. When comparisons were made on the basis of rat units, estrone was more effective than estradiol in the bird.

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# THE FORMATION OF PHOSPHATIDES IN THE ORGANISM UNDER NORMAL AND PATHOLOGICAL CONDITIONS\*

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Investigations carried out during the last few years with radioactive phosphorus  $P_{15}^{32}$  as indicator have clearly shown that the amounts of phosphatides newly formed from inorganic phosphate during a given period vary considerably for different organs (1-4): the highest percentage of labeled phosphatides is found in the liver and in the intestinal tract; the kidneys which are somewhat less active are next in order, followed by the eviscerated carcass and the brain. In a recent study from this laboratory (4) it was demonstrated that the intestinal tract and the liver contained considerably more newly formed lecithin than cephalin,<sup>1</sup> and the possibility of the formation in the body of cephalin from lecithin was discussed.

The important question as to the seat and the mechanism of formation of the phosphatides in the organism made a study of the influence of partial hepatectomy on these processes desirable. The present paper combines a report on this problem with a continued investigation of the relative speed of synthesis of lecithin and cephalin in various organs of the normal organism.

## EXPERIMENTAL

### *Material and Methods*

The radioactive phosphorus  $P_{15}^{32}$  used was prepared by deuteron bombardment in the cyclotron. In one experimental series (Rats

\* This work has been supported by a grant from the John and Mary R. Markle Foundation.

<sup>1</sup> The same relation appears to hold true for tumor tissue (5).



H1 and H2) radioactive phosphorus of much lower radioactivity was employed which had been prepared by the action of fast neutrons on carbon disulfide (4). The material was administered in the form of a neutral aqueous solution of  $\text{Na}_2\text{H}^*\text{PO}_4$ .<sup>2</sup>

The radioactivity of the various phosphatide fractions isolated was measured in the dry state by means of a Geiger-Müller counter as described previously (6, 4). It is expressed in KF units per mg. of P.

The methods for the extraction and isolation of the phosphatides were, with the exceptions noted below, essentially the ones described in a preceding publication (4).

#### *Formation of Phosphatides in Rabbit*

In order to obtain the organ phosphatides in amounts sufficient for a more rigorous purification than is possible with the phosphatides from one rat, the relative speed of formation of lecithin and cephalin in the various organs of the rabbit was studied. The animal (1550 gm.) received 144.1 mg. of  $\text{Na}_2\text{H}^*\text{PO}_4$ , dissolved in 6 cc. of water, by subcutaneous injection; 6 hours later a second equal dose was injected. The total amount administered corresponded, therefore, to 63.0 mg. of  $^*\text{P}$  with an activity (calculated on the same basis as the values given in Table I) of 89,500,000 KF units. The animal received no food during the experiment.

24 hours after the first injection some blood was obtained from the animal by heart puncture. It then was killed and the liver, kidneys, intestinal tract, and brain were removed. In all experiments here described the intestinal tracts were washed with water before the extraction of the lipids. The organs and a portion of the skinned eviscerated carcass were mechanically ground, dehydrated, and extracted by the method outlined previously (4).

The *lecithin fractions* were in all cases, after removal of the alcohol-insoluble *cephalin*, converted into the double salts with  $\text{CdCl}_2$  which were washed with cold ether and recrystallized from ethyl acetate-alcohol. The lecithin fractions were recovered in the usual manner after decomposition of the  $\text{CdCl}_2$  double salts with a 20 per cent solution of dry ammonia in methyl alcohol.

<sup>2</sup> The asterisk before the symbol for an element indicates an unstable isotope.

In this experiment the brain protagon fraction was also isolated in order to obtain information about the rate of *sphingomyelin* formation. For this purpose the brain powder after removal of lecithin and cephalin with alcohol-ether was extracted with hot chloroform. To this extract the small amount of material insoluble in cold petroleum ether which had been obtained by chilling the solution of brain lecithin and cephalin in petroleum ether (cf. (4) p. 590) was added. The cerebroside-sphingomyelin mixture, prepared from the concentrated chloroform solution by precipitation with ether and reprecipitation with acetone, weighed 21.1 mg. and contained 1.9 per cent P.

From 26 cc. of blood (obtained by cardiac puncture) to which potassium oxalate had been added the blood cells were removed by centrifugation. The cells, dried with acetone and extracted with alcohol-ether in the usual manner, yielded only 1.3 mg. of mixed blood cell phosphatides.

The results of this experiment are summarized in Table I. The mixed phosphatides from the blood cells had an activity of 250 KF units per mg. of phosphatide. The extremely small amount obtained prevented the performance of a phosphorus analysis, but on the assumption of a P content of between 3 and 4 per cent an activity value of 6300 to 8300 KF units per mg. of \*P would result. As a comparison with the values given in Table I will show, this places the blood cells among the least active systems of the organism with regard to lipid turnover.

#### *Formation of Phosphatides in Rat*

In a preceding study (4) the relative speed of synthesis of lecithin and cephalin in the various organs of the rat was investigated with animals which had received radioactive phosphate of very low activity and had been kept on the ordinary laboratory diet during the utilization of the labeled phosphate. For purposes of comparison with the experiments on partially hepatectomized rats which will be reported later in this paper it was desirable to obtain data on the utilization of ingested radioactive phosphate by the fasting rat.

An adult albino rat (300 gm.) received 24.0 mg. of  $\text{Na}_2\text{H}^*\text{PO}_4$ , dissolved in 1 cc. of water, by stomach tube. 6 hours later a second equal dose was administered. The total amount fed cor-

responded to 10.5 mg. of \*P with an activity (calculated on the same basis as the values given in Table II) of 24,280,000 KF units. The animal which had fasted during the experiment was killed

TABLE I  
*Relative Speed of Phosphatide Synthesis in Fasting Rabbit*

Organ	Phosphatide	Weight	P	Radio-activity* in 1 mg. *P	Minimum amount of newly formed phosphatide, in per cent of total phosphatide
		mg.	per cent	KF units	
Intestinal tract	Lecithin	128.1	3.3	171,800	12.1
	Cephalin	314.6	2.9	107,300	7.5
Liver	Lecithin	171.4	3.7	136,800	9.6
	Cephalin	338.6	3.1	95,300	6.7
Kidney	Lecithin	22.5	3.5	69,000	4.9
	Cephalin	8.7	3.0	62,240	4.4
Brain	Lecithin	109.2	3.7	1,414	0.10
	Cephalin	72.1	4.0	2,030	0.14
	Sphingomyelin	21.1	1.9	2,390	0.17
Carcass	Lecithin	542.1	3.4	16,900	1.2
	Cephalin	409.0	2.9	21,820	1.5

\* The spread of the individual counts was within about 3 per cent of the mean values given.

TABLE II  
*Relative Speed of Phosphatide Synthesis in Fasting Rat*

Organ	Phosphatide	Weight	P	Radio-activity* in 1 mg. *P	Minimum amount of newly formed phosphatide, in per cent of total phosphatide
		mg.	per cent	KF units	
Liver	Lecithin	106.8	3.5	174,600	7.5
	Cephalin	18.0	3.5	148,900	6.4
Intestinal tract	Lecithin	26.0	3.2	123,000	5.3
	Cephalin	9.2	3.4	90,500	3.9
Kidney	Lecithin	19.7	3.5	52,170	2.3
Brain	"	29.3	3.8	2,013	0.09
	Cephalin	14.8	3.6	6,165	0.27
Carcass	Lecithin	557.5	3.1	18,020	0.78
	Cephalin	298.8	2.9	15,220	0.66

\* The spread of the individual counts was within about 3 per cent of the mean values given.

24 hours after the administration of the first dose. The organs were removed and examined in the usual manner. The results of the experiment are contained in Table II. The cephalin fractions from the kidneys are not included, since the amounts obtainable from one animal (between 1 and 2 mg.) were too small for accurate analysis.

### *Formation of Phosphatides in Partially Hepatectomized Rats*

The operations were carried out on adult albino rats. The technique for the partial excision of the liver was in general similar to that described by Higgins and Anderson (7) and by Brues

TABLE III  
*Partially Hepatectomized Rats*

Rat No.	Body weight before operation	Liver portion removed	Liver remnant 48 hrs. after operation	Estimated per- centage of liver removed by operation
	gm.	gm.	gm.	
H1	320	7.8	7.1	68
H2	345	7.1	6.6	58
H3	302	5.1	6.8	47
H4	325	5.5	7.4	47

*et al.* (8). The data on the animals used are summarized in Table III. From Rats H3 and H4 the entire left lateral lobes and somewhat more than half of the median lobes were removed. A slightly larger portion of the liver was excised from Rats H1 and H2 according to the technique of Brues *et al.* (8). The estimated percentage of liver removed given in the last column of Table III is an approximation based on the report of Higgins and Anderson (7) that in their observations the livers averaged 3.58 per cent of the body weights.

The liver remnants of all animals used had the appearance of fatty livers. This is in agreement with the observations of Collip *et al.* (9).

24 hours after the removal of part of the liver the animals received a solution of  $\text{Na}_2\text{H}^*\text{PO}_4$  by stomach tube. They were given no food during the experiment, but had free access to drinking water. The animals were killed 24 hours after the adminis-

TABLE IV

*Relative Speed of Phosphatide Synthesis in Partially Hepatectomized Rats*

Rat No.	Organ	Phosphatido	Weight	P	Radio-activity* in 1 mg. *P	Minimum amount of newly formed phosphatide, in per cent of total phosphatide
			mg.	per cent	KF units	
H1	Liver	Lecithin	118.9	3.3	356	1.5
		Cephalin	18.1	2.8	285	1.2
	Intestinal tract	Lecithin	52.1	2.9	263	1.1
		Cephalin	7.2	2.5	†	†
	Kidney	Lecithin	40.0	3.5	286	1.2
	Brain	"	44.1	3.2	†	†
		Cephalin	19.4	2.9	†	†
	Carcass	Lecithin	816.0	3.1	56	0.24
		Cephalin	306.8	2.9	36	0.16
H2	Liver	Lecithin	97.0	3.5	189	0.81
		Cephalin	17.2	2.6	163	0.70
	Intestinal tract	Lecithin	53.3	3.0	163	0.70
		Cephalin	4.2	2.4	†	†
	Kidney	Lecithin	39.1	3.5	118	0.51
	Brain	"	45.4	3.2	†	†
		Cephalin	17.7	2.7	†	†
	Carcass	Lecithin	982.0	3.1	20	0.09
		Cephalin	422.1	3.3	25	0.11
H3	Liver	Lecithin	80.7	3.6	6,482	1.3
		Cephalin	7.4	3.4	7,437	1.4
	Intestinal tract	Lecithin	41.4	3.2	6,890	1.3
		Cephalin	3.6	3.0	6,621	1.3
	Kidney	Lecithin	21.6	3.6	4,726	0.92
	Brain	"	38.5	3.4	204	0.04
		Cephalin	6.8	3.9	525	0.10
	Carcass	Lecithin	49.4	3.2	1,345	0.26
		Cephalin	187.0	2.9	697	0.14
H4	Liver	Lecithin	55.0	3.4	11,580	2.3
		Cephalin	7.5	2.8	9,035	1.8
	Intestinal tract	Lecithin	38.6	2.9	8,928	1.7
		Cephalin	4.2	2.8	6,592	1.3
	Kidney	Lecithin	22.2	3.7	7,898	1.5
	Brain	"	48.5	3.3	275	0.05
		Cephalin	7.5	3.9	882	0.17
	Carcass	Lecithin	136.0	2.8	1,968	0.38
		Cephalin	206.4	3.0	947	0.18

\* The spread of the individual counts was within about 3 per cent of the mean values given.

† No measurable activity.

tration of the labeled sodium phosphate and examined in the usual manner.

Rats H1 and H2 each received 23.6 mg. of  $\text{Na}_2\text{H}^*\text{PO}_4$ , dissolved in 1 cc. of water by stomach tube; *i.e.*, in each case a total of 5.15 mg. of  $^*\text{P}$  of comparatively low radioactivity, *viz.* 119,500 KF units. Rats H3 and H4 each received 96.0 mg. of  $\text{Na}_2\text{H}^*\text{PO}_4$ , dissolved in 2 cc. of water; *i.e.*, in each case a total of 21.0 mg. of  $^*\text{P}$  with an activity of 10,800,000 KF units. These activity data are computed on the same basis as the results of both experimental series which are summarized in Table IV.

#### DISCUSSION

The results reported in the first part of this publication concerning the relative speed of formation of lecithin and cephalin in the various organs of the body fully confirm the findings contained in a previous paper (4). In the intestinal tract and in the liver the amount of newly formed lecithin considerably exceeds that of new cephalin. The number of molecules of both phosphatide types synthesized in different organs during the experiment decreases in the same order; *viz.*, liver, intestinal tract, kidneys, eviscerated carcass, brain. The amounts of newly formed phosphatides obtained from the liver and the intestinal tract are of the same order of magnitude, the liver usually containing somewhat more. Whether the reversal of this order in the rabbit experiment reproduced in Table I is of significance cannot be said.

There appears to be one organ in which more new cephalin is found than lecithin, namely the brain. This organ shows an extremely low metabolic activity with regard to the phosphatides, if the rate at which radioactive phosphorus appears in its fractions is taken as a measure. With highly radioactive phosphate preparations, however, as used in most of the experiments here discussed, it is possible to measure the radioactivity of the brain lipids with sufficient accuracy. An inspection of the results reproduced in Tables I, II, and IV shows that the brain cephalin in practically all cases had a somewhat higher radioactivity than had the brain lecithin fractions. These results, if significant, would point to a certain autonomy of the central nervous system with regard to lipid metabolism. The small amount of newly formed sphingomyelin found in the preparation from rabbit brain (Table I) brings this phosphatide in line with the other brain phospholipids.

For a consideration of the effect of partial hepatectomy on the phosphatide metabolism it is perhaps advisable first to compute the percentage of radioactive P fed which was recovered in the form of phosphatides in the various cases. From the data on the radioactivity of the different fractions contained in Table IV it can be calculated that approximately 3.4, 1.7, 0.4, and 0.5 per cent of the radioactive phosphorus administered were recovered from Rats H1, H2, H3, and H4 respectively in the form of purified phosphatides. This has to be compared with recoveries of 4.7 per cent (Table I) and 5.6 per cent (Table II) in the normal animals examined.

The first conclusion to be drawn from these experiments, therefore, is that the ability of the organism to synthesize phosphatides is to a certain extent impaired by the removal of a portion of the liver. Whether this relative impairment extends to phosphorus metabolism in general is uncertain. It may be that during the period of liver regeneration which is known to proceed at an extraordinary rate ((7, 8); *cf.* also (10, 11)) a larger proportion of the available phosphorus compounds is mobilized for restorative tasks.

The second conclusion made possible by these experiments is that the rate at which new phosphatide molecules are synthesized by the various organs is more affected by hepatectomy in those organs which normally have the most active lipid metabolism (liver and intestinal tract) than in the rest of the organism.

The third conclusion is that the profound disturbance of phosphatide metabolism produced by the partial removal of the liver is accompanied by a blurring of the normal differences between the rates at which lecithin and cephalin are synthesized in the liver and the intestinal tract.

It should, in this connection, be remembered that choline has been found to be without effect on the fatty livers produced by partial hepatectomy (12). Since, whenever choline is to be utilized for the synthesis of phospholipids, esterification with phosphoric acid will have to take place in one of the phases of the reaction, the failure of choline to influence the fatty livers due to partial hepatectomy, together with the effect of this operation on the phosphatide metabolism discussed here, seems to point to a decreased ability of the injured liver to effect phosphorylation. It has been pointed out elsewhere (13) that the metabolism of

cholinephosphoric acid as well as that of free choline will have to be considered for a proper formulation of the mechanism of the disturbances of lipid metabolism.

The experiments presented here are in harmony with the views expressed in a previous communication (4) concerning the possible mechanism of formation of the body phosphatides. If a common origin for the two main phosphatide types is to be assumed, the formation in the organism of cephalin from lecithin by demethylation appears plausible. The opposite reaction, *viz.* the physiological methylation of cephalin to form lecithin, is highly unlikely on the basis of the results described elsewhere (4), as well as of those presented in the first part of this paper. As corroborative evidence the inability of the body to convert ethanolamine into choline may be cited, as shown by the failure of ethanolamine to replace choline in the prevention of fatty livers (14). Of interest in this connection is the fact that ethanolamine has been found unable to support growth when homocystine instead of methionine is fed (15).

It is not possible here to go into the question of the function and of the seat of formation of the body phosphatides. Discussions of various phases of this topic will be found in papers cited (2, 16-18) and in several recent papers from this laboratory. It appears likely that phosphatides are synthesized in the body in a number of places simultaneously. The results of the experiments concerning the effect of partial hepatectomy on the formation of phosphatides discussed above would seem to point in this direction.

The authors are highly indebted to Dr. J. R. Dunning of the Department of Physics of this University and to Dr. E. O. Lawrence of the Radiation Laboratory of the University of California for the radioactive phosphorus used in these experiments. The assistance rendered by Mr. B. Krcs and Mr. A. Bendich is gratefully acknowledged.

#### SUMMARY

The relative speed of formation of lecithin and cephalin in various organs of normal and partially hepatectomized animals was examined by means of the radioactive phosphorus isotope. The removal of part of the liver produced a considerable disturbance



of phosphatide metabolism. Some of the biological implications of the findings are discussed.

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# THE METABOLISM OF AMINOETHYLPHOSPHORIC ACID, FOLLOWED BY MEANS OF THE RADIO-ACTIVE PHOSPHORUS ISOTOPE\*

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Aminoethylphosphoric acid,  $\text{NH}_2\text{CH}_2\text{CH}_2\text{OPO}(\text{OH})_2$ , was first isolated from bovine malignant tumors by Outhouse (1). Since benign tumors and a number of normal tissues did not yield this compound, it was thought to be specific for malignant tumor tissue. Recently, however, Colowick and Cori (2) reported the isolation of the acid from the small intestine of rabbits and pigs.

The possible relationship of this compound to the metabolism of phosphatides, particularly to that of cephalin, is obvious. It may be significant that it appears to be present in largest amounts in tissues in which the phosphatide metabolism is extremely vigorous.

The biological function of aminoethylphosphoric acid could be that of an intermediate in the synthesis of phosphatides by the organism, or that of a breakdown product of the phosphatides. Very little is known about the mechanism by which phospholipids are formed in the body. A study of the utilization of aminoethylphosphoric acid appeared, therefore, of interest. For this purpose a preparation was employed which contained the radioactive phosphorus isotope  $\text{P}_{15}^{32}$ . The present paper gives a report on the conversion of this compound into phosphatides in different organs. Some data on tumor-bearing animals are included.

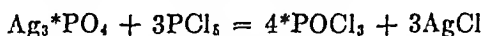
## EXPERIMENTAL

### *Synthesis of Radioactive Aminoethylphosphoric Acid*

A method for the preparation of radioactive phosphorus oxychloride, involving the conversion of  $^*\text{P}$  into  $^*\text{PCl}_3$  and the oxida-

\* This work has been supported by a grant from the John and Mary R. Markle Foundation.

tion of the latter to  $^*\text{POCl}_3$ , has been briefly reported elsewhere (3).<sup>1</sup> In the present work it was prepared from radioactive phosphate by means of the following reaction.



Radioactive sodium phosphate was converted into the silver salt. To 6.89 gm. of dry  $\text{Ag}_3^*\text{PO}_4$  (0.016 mole) in a bomb tube 11.2 gm. of powdered  $\text{PCl}_5$  (0.054 mole) were added. The sealed tube was heated to  $130^\circ$  for 10 minutes. The  $^*\text{POCl}_3$  which had formed at that time was refluxed for a few minutes by careful heating with a microburner in order to break up the  $\text{AgCl}$  lumps. The *radioactive phosphorus oxychloride* was finally purified by distillation *in vacuo* into a trap cooled with solid  $\text{CO}_2$ . It weighed 8.15 gm. (yield, 83 per cent).

The barium salt of *radioactive aminoethylphosphoric acid* was synthesized according to Outhouse (1).

<i>Analysis</i> — $\text{C}_2\text{H}_6\text{O}_4\text{N}^*\text{PBa}$ .	Calculated.	P 11.2, N 5.0
276.4	Found.	" 11.4, " 4.8

For the metabolism experiments this preparation was converted into the sodium salt.

### Methods

The radioactivity of the various phosphatide fractions discussed later in this paper was determined in the dry state by means of a Geiger-Müller counter according to the method generally used in this laboratory (4, 5). The urine samples were concentrated, and the phosphorus, after acid digestion, precipitated as the ammonium molybdate complex. The weighed precipitates were dissolved in  $\text{NaOH}$  solution and their radioactivity was determined in solution by means of the technique previously described (4). The amount of radioactive phosphorus accumulated in the livers and tumors of the tumor-bearing animals was likewise determined in solution after digestion of weighed tissue portions with a mixture of nitric and sulfuric acids. All activities are expressed in KF units per mg. of  $^*\text{P}$  (4).

As usual, all radioactivity measurements were accompanied by

<sup>1</sup> The asterisk before the symbol for an element indicates an unstable isotope.

standard measurements in order to correct for the decay of the unstable phosphorus administered to the animals and to express the activity counts in terms of the radioactive preparation employed. The standards consisted (a) for the measurements in the dry state, of a suspension of the radioactive aminoethylphosphate preparation in vegetable lecithin, (b) for the measurements on the phosphomolybdate solutions, of a similar solution of the ammonium phosphomolybdate complex prepared from the aminoethylphosphate, (c) for the measurements on the acid digests of organs, of an aqueous solution of the aminoethylphosphate.

The methods for the extraction and isolation of the phosphatides were essentially the same as in previous publications (5, 6).

### *Metabolism of Aminoethylphosphoric Acid*

Two adult rats (body weight 270 and 295 gm. respectively) each received 80 mg. of disodium aminoethylphosphate, dissolved in 2 cc. of water, by subcutaneous injection; *i.e.*, in each case a total of 13.4 mg. of  $^3\text{P}$  with an activity of 300,000 KF units (calculated on the same basis as the figures given in Table I). The animals, which received no food for 24 hours prior to and during the experiments, were killed 24 hours after the administration of the radioactive material and examined in the usual manner. The results of the experiment are summarized in Table I. The lecithin and cephalin fractions of the brain are not included, since they showed no measurable radioactivity. As stated before (6) the amount of cephalin obtainable from the kidneys of one rat does not suffice for activity measurement and analysis. Data on the amount of  $^3\text{P}$  excreted in the urine will be found later in this paper.

### *Utilization of Aminoethylphosphoric Acid by Tumor-Bearing Animals*

A few orienting experiments were carried out concerning the metabolism of aminoethylphosphoric acid in tumor-bearing rats. Two adult rats with well developed carcinomas<sup>2</sup> (body weight 282

<sup>2</sup> Carcinoma of the rat breast (No. 2426) which had been transplanted into animals of the inbred strain in which the tumor arose. We wish to thank Dr. W. H. Woglom of the Institute of Cancer Research of this University for the animals used.

TABLE I  
*Formation of Phosphatides from Aminoethylphosphoric Acid*

Rat No.	Organ	Phosphatide	Weight	P	Radioactivity* in 1 mg. *P	Minimum amount of newly formed phosphatide, in per cent of total phosphatide
			<i>mg.</i>	<i>per cent</i>	<i>KF units</i>	
1	Liver	Lecithin	120.3	3.9	1630	7.3
		Cephalin	14.9	3.7	780	3.5
	Intestinal tract	Lecithin	13.7	3.2	800	3.6
		Cephalin	21.2	2.9	690	3.1
2	Kidney	Lecithin	19.2	3.8	590	2.6
	Liver	"	124.3	3.5	1470	6.5
		Cephalin	8.0	3.6	870	3.9
	Intestinal tract	Lecithin	30.8	3.3	940	4.2
		Cephalin	12.6	3.0	420	1.9
	Kidney	Lecithin	21.7	3.6	620	2.8

\* The spread of the individual counts was within about 3 per cent of the mean values given.

TABLE II  
*Accumulation of \*P Originating from Aminoethylphosphoric Acid in Tumor Rats*

Organ	*P in 1 gm. fresh tissue	
	Rat 3	Rat 4
	<i>mg.</i>	<i>mg.</i>
Liver.....	0.0601	0.0680
Tumor.....	0.0365	0.0431

TABLE III  
*Phosphatides from Tumor Rats*

Rat No.	Organ	Weight	P	Radioactivity* in 1 mg. *P	Minimum amount of newly formed phosphatide, in per cent of total phosphatide
		<i>mg.</i>	<i>per cent</i>	<i>KF units</i>	
3	Liver	24.5	3.4	1490	6.6
	Tumor	4.2	3.2	1190	5.3
4	Liver	4.9	3.4	1350	6.0
	Tumor	26.8	3.4	960	4.3

\* The spread of the individual counts was within about 3 per cent of the mean values given.

and 223 gm. respectively) received the same amount of radioactive aminoethylphosphoric acid as the normal animals discussed in the preceding paragraph; *i.e.*, each animal was given a subcutaneous injection of 13.4 mg. of  $^3\text{P}$  with an activity of 300,000 KF units, contained in 2 cc. of water. The animals, which were fasted for 24 hours prior to and during the experiment, were killed 24 hours after the administration of the radioactive material. The livers and tumor tissues were removed. A portion of each organ was destroyed with acid and the total accumulation of  $^3\text{P}$  determined by measurement of the radioactivity, as described above; the phosphatides were prepared from the remaining tissue in the usual manner. The amounts of  $^3\text{P}$  originating from the radioactive

TABLE IV  
*Excretion of  $^3\text{P}$  in Urine after Administration of Radioactive Aminoethylphosphoric Acid*

Rat No.	Collection of specimen; time after administration of radioactive material	Total P excreted	$^3\text{P}$ excreted	$^3\text{P}$ in total P excreted
	hrs.	mg.	mg.	per cent
1	8	12.5	3.91	31.2
	18	9.1	0.67	7.4
2	8	11.7	3.93	33.7
3	8	9.1	3.27	35.9
4	8	*	*	33.6

\* Partly lost by accident.

aminoethylphosphate which were contained in the liver and tumor tissue 24 hours after the administration of the material are compared in Table II.

The relative activities of the phosphatide preparations from the tumor and liver of the animals are given in Table III. These values are strictly comparable to the figures given in Table I. No attempt was made in this case to separate the ether-soluble phosphatides into their components, since only portions of the tissues were available for examination.

#### *Excretion of Radioactive Phosphorus in Urine*

The excretion of  $^3\text{P}$  in the urine specimens collected within 8 hours after the administration of radioactive aminoethylphos-

phoric acid was followed in all animals. The preparation of the material for the activity measurements has been described above. In the case of Rat 1 a subsequent 10 hour specimen was likewise examined. The results of this experiment are summarized in Table IV.

#### DISCUSSION

The most striking result of the experiments reported here is that the body apparently is unable to utilize aminoethylphosphoric acid as such for the synthesis of cephalin. The relative amounts of lecithin and cephalin newly formed in the liver and the intestinal tract, given in Table I, indicate that the new lecithin exceeded the new cephalin to an even higher degree than when inorganic phosphate was administered (5, 6).

The utilization of aminoethylphosphoric acid for the synthesis of phosphatides in the body could proceed along one of the following lines: (1) Aminoethylphosphoric acid combines with a diglyceride to form cephalin. (2) Aminoethylphosphoric acid is first methylated to cholinephosphoric acid which in turn combines with a diglyceride to form lecithin. (3) Aminoethylphosphoric acid is first split into ethanolamine and phosphoric acid, and the latter used for the synthesis of lecithin. Reaction 1 is made extremely unlikely by the experiments here described in which much larger amounts of newly formed lecithin than cephalin were found. Reaction 2 cannot be excluded on the basis of the present experiments, although the failure of ethanolamine to replace choline as lipotropic (7) or dietary (8) agent would seem to speak against it.<sup>3</sup> The most likely assumption, for the time being, is that expressed in Reaction 3. Enzymes which hydrolyze aminoethylphosphoric acid have been found in kidney and feces (9), and the occurrence in liver of an enzyme which splits cholinephosphoric acid has been made probable (10). The simplest series of reactions would, therefore, appear to be enzymatic hydrolysis of aminoethylphosphoric acid in the tissues, utilization of the inorganic phosphate for the synthesis of lecithin, demethylation of

<sup>3</sup> Under the conditions of the experiments the amount of methionine available to the animals must have been very small. It would be of interest to determine whether in the presence of a methyl group donor like methionine more lecithin is formed from aminoethylphosphoric acid.

lecithin to form cephalin. The aminoethylphosphoric acid normally occurring in the body tissues probably is the product of the catabolism of cephalin.

One other point appears noteworthy; *viz.*, the large amount of radioactive phosphorus excreted in the urine during the first 8 hours of the experiment (Table IV). In this time more than a quarter of the radioactive phosphorus administered had passed through the kidneys. There was no difference in this respect between the normal and tumor-bearing animals. As can be seen, a high percentage, about one-third, of the total phosphorus excreted was radioactive phosphorus which came from the aminoethylphosphoric acid. During the next 10 hours the concentration of radioactive phosphorus dropped to 7 per cent of the total phosphorus eliminated.

The experiments on the metabolism of aminoethylphosphoric acid in tumor-bearing animals provide no basis for the assumption of a specific function of this compound in malignant growth. The liver showed a faster rate of phosphatide turnover and of total phosphorus uptake than the tumor (Tables II and III). A comparison of Tables I and III will, however, show that tumors belong to the most active tissues with regard to the formation of phosphatides from aminoethylphosphoric acid. This has been demonstrated before, as far as the utilization of inorganic phosphate is concerned (11, 12).

The authors would like to express their gratitude to Dr. J. R. Dunning of the Department of Physics of this University and to Dr. E. O. Lawrence of the Radiation Laboratory of the University of California for the radioactive phosphorus used in the experiments here described. They are indebted to Mr. B. Kress for general assistance.

#### SUMMARY

Experiments on the metabolism of radioactive aminoethylphosphoric acid indicate that this compound is not directly utilized for the synthesis of cephalin. After its administration to rats more newly formed lecithin than cephalin is found both in the liver and the intestinal tract. The bearing of the findings on the theory of phosphatide metabolism in the body is discussed.



Experiments with tumor-bearing animals fail to demonstrate a specific function of aminoethylphosphoric acid in malignant growth. It is considered as a normal breakdown product of cephalin.

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# THE NUCLEIC ACID CONTENT AND DISTRIBUTION IN *STREPTOCOCCUS PYOGENES*\*

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Nucleic acids conjugated with proteins are an essential part of the nuclei of all animal and plant cells (2). They are present so far as is known in all bacteria and viruses; indeed the smaller viruses, which appear to be the simplest entities having the essential attributes of life (3), appear to be pure nucleoproteins (4).

Nucleic acids, which exhibited serological reactivity by giving precipitates with certain antipneumococcal horse sera and with one rabbit antistreptococcal serum, have been isolated for the first time from  $\beta$ -hemolytic streptococci of Lancefield's Group A (*Streptococcus pyogenes*) (5, 6). While the nature of this serological activity of nucleic acids is being investigated, we present here some data on their distribution in streptococci of several types and variant phases.

## *Determination of Nucleic Acids in Streptococci*

At the start of this work a satisfactory method for the quantitative determination of nucleic acids in bacteria was lacking. Attempts to extract the nucleic acids with dilute alkali were not only incomplete but also involved tedious and complicated manipulations. Streptococci washed three times with distilled water were dried and total phosphorus was determined by the colorimetric method of King (7), the purine nitrogen by the method of Graff and Maculla (8), and total nitrogen by the micro-Kjeldahl method. These analytical results were irregular, as values varied from cul-

\* This work has been supported by a grant from the Commonwealth Fund. It was communicated to the Third International Congress of Microbiology, New York, September, 1939 (1).

ture to culture. It was evident that in washing the centrifuged culture sediments with distilled water, extraneous materials, such as inorganic phosphates, were not completely eliminated.

With the above procedure the average of the duplicate analyses of each of six different washed and dried culture sediments of Type 6 (Strain 1048M) streptococci gave the following range of results: 1.65 to 2.34 per cent phosphorus, 1.15 to 2.05 per cent purine nitrogen, 11.1 to 14.3 per cent total nitrogen. Analytical results of the other types of organisms within Group A showed similar irregularities.

*Extraction of Streptococci with Acidulated Alcohol*—The above preliminary experiments convinced us that the fresh culture sediments contained a varying amount of certain phosphorus compounds (organic and inorganic) in excess of the nucleic acid phosphorus, and varying amounts of other inorganic salts. The bacterial sediment from 5 liters of broth culture grown for 16 hours was washed once with distilled water, then suspended in 250 cc. of 95 per cent alcohol which contained 0.5 cc. of concentrated hydrochloric acid, and shaken mechanically for 30 minutes. The sediment was then centrifuged and the above treatment repeated twice. After the third washing the sediment was extracted with ether and dried, first at 56°, and then at 110°, before analysis for phosphorus, purine nitrogen, and total nitrogen.

*Analysis of Alcoholic Extracts*—The acidulated alcoholic extracts were neutralized and evaporated to dryness over a water bath. The residues were taken up in a volume of 25 cc. of distilled water and analyzed for total and inorganic phosphorus, and total nitrogen. In two experiments with two different cultures of Type 6 (Strain 1048M) streptococci, respectively 1.25 and 1.60 mg. of nitrogen, and 0.74 and 0.67 mg. of total phosphorus were found in the extract, per 100 mg. of dry organisms extracted as described. Of these phosphorus values 0.4 and 0.37 mg., respectively, were inorganic phosphorus. In two similar experiments alcoholic extracts of Type 1 (Strain 1685M) organism respectively contained 1.42 and 1.87 mg. of nitrogen and 0.51 and 0.63 mg. of total phosphorus per 100 mg. of extracted organisms. Of this phosphorus 0.31 and 0.36 mg., respectively, were inorganic phosphorus.

Therefore it appeared that the organisms washed with distilled water contained acid alcohol-extractable material which in Strain

1048M had 1.25 to 1.60 per cent N, and in Strain 1685M had 1.42 to 1.87 per cent N.

The alcoholic extract on evaporation to dryness at 110° without the neutralization of the acid yielded total solids ranging between 10 and 12 per cent. The extracted material on drying at 110° assumed a charred, dark brown appearance and had a pungent odor. The data thus obtained with four different strains are given in Table I.

*Analysis of Streptococci Extracted with Acidulated Alcohol*—The extracted organisms were dried at 110° and 8 to 10 mg. were used for the determination of phosphorus, 10 to 12 mg. for total nitrogen, and about 20 mg. for purine nitrogen. Determinations on

TABLE I  
*Alcoholic Extraction of Streptococci*

Strain No.	Weight of extracted whole organisms dried at 110°	Weight of extract dried at 110°	Extractable material in whole organisms
	mg.	mg.	per cent
1048M	1045	142	12
1685M	455	61	12
C203R	250	28	10
B <sub>2</sub> R	500	53	10

At present we are not in a position to state how much of the extractable material is actually a part of the whole organism and therefore we have not made any correction for this material in the data presented in Table II.

each sample were carried out in duplicate, and the values reported constituted the averages of the duplicate determinations, which in all cases tabulated agreed with each other within 3 per cent. From the values presented in Table II it can be seen that the ratio of purine nitrogen to phosphorus approximated that of the theoretical ratio for nucleic acids.

In presenting this observation we are not assuming that all the phosphorus in streptococci is present in nucleic acid. In fact we have obtained from streptococci a high phosphorus-containing non-nucleic acid fraction. However, the amount of this fraction is very low and therefore will not seriously affect the data of Table II.

*Determination of Desoxyribose Nucleic Acid in Streptococci*—It

is well known that certain cells and bacteria contain both the *d*-ribose and desoxyribose types of nucleic acids (9). In this laboratory Zittle (10) showed that neutral extracts of  $\beta$ -hemolytic

TABLE II

*Nucleic Acid and Nucleoprotein Content of Group A Streptococci*

Strain	Colony characteristics	Total N	Total P	Purine N	Ratio, purine N to P	Nucleic acid, average*	Protein or nucleoprotein†
		per cent	per cent	per cent		per cent	per cent
Thymus (desoxyribose) nucleic acid (theory)			9.89	11.17	1.13		
Yeast ( <i>d</i> -ribose) nucleic acid (theory)			9.64	10.74	1.11		
1048M	Mucoid	13.5	1.85	2.14	1.16	20.4	84.3
		13.5	1.89	2.21	1.17		84.3
1685M	"	13.3	2.18	2.35	1.08	22.5	83.1
		13.6	2.46	2.59	1.05		85.0
1685S	Smooth	13.2	2.11	2.15	1.02	20.8	82.5
		13.1	2.18	2.41	1.11		81.8
C203M	Mucoid	13.2	2.25	2.17	0.97	20.7	82.5
		13.7	2.42	2.36	0.98		85.5
C203S	Smooth	13.7	2.10	2.32	1.10	20.2	85.5
		13.6	1.86	2.07	1.11		85.0
C203R	Rough	13.5	1.84	1.76	0.96	16.8	84.3
		13.6	1.64	1.92	1.16		85.0
B <sub>3</sub> S	Smooth	13.3	2.21	2.66	1.19	24.1	83.1
		13.1	2.21	2.62			81.8
B <sub>3</sub> R	Rough	13.4	1.26	1.66	1.29	14.8	83.8
			1.30	1.58			
NY5M	Mucoid (small)	13.5	1.85	2.14	1.14	19.3	84.4
				2.08			
NY5R	Rough	12.7	1.56	1.65	1.04	14.8	79.4
		12.5		1.58			78.2
10-1-S	Smooth	13.5	1.75	1.70	1.01	16.2	84.3
		13.7	1.78	1.85			85.5
10-2-S	"	13.5	1.76	1.90	1.11	18.3	84.3
		13.2	1.83	2.00			82.5
10-2-R	Rough	12.2	1.42	1.48	1.12	15.1	76.2
		12.4	1.54	1.74			77.5
		12.0		1.77			75.0

These data have not been corrected for extractable material for the reason stated at the bottom of Table I.

\* Based on purine nitrogen.

† Based on total nitrogen  $\times 6.25$ .

streptococci gave color reactions for both types of nucleic acids. In this study we have attempted to evaluate roughly the per cent of desoxyribose type of nucleic acid in the extracted streptococci as follows:

4 cc. of 0.1 N HCl were added to 25 to 35 mg. of dried and ground whole organisms. This mixture was placed in a boiling water

TABLE III  
*Biological Properties of Strains Analyzed*

Strain No.	Colony form*	Mouse virulence†	Nature of agglutination reaction	Griffith's type No.
1048M	M	10 <sup>-7</sup> to 10 <sup>-8</sup>	Type-specific	6
1685M	Ms	10 <sup>-7</sup> " 10 <sup>-8</sup>	"	1
1685S	S	Avirulent	"	1
C203M	M	10 <sup>-4</sup>	Group and type	1
C203S	S	Avirulent	" " "	1
C203R	R	"	" " very small amount of Type 1	
B <sub>1</sub> S	Sm	" (12)		
B <sub>1</sub> R	Rs	" (12)		
NY5 Wadsworth	Ms	10 <sup>-8</sup>	Type-specific	10
NY5R	R	Avirulent		
10-1-S	S	" ‡	Group-specific	
10-2-S	"	" ‡	Type-specific	5
10-2-R	R	" ‡	"	5

\* M = mucoid, Ms = mucoid with a slight tendency toward smooth, Sm = smooth with traces of mucoid, S = smooth, Rs = rough with traces of smooth, R = rough.

† The figures indicate the approximate amount of an 18 hour blood broth culture required to kill a white mouse in 24 to 48 hours. The figures in parentheses refer to the bibliography.

‡ Hadley and Hadley (13) and personal communication.

bath and hydrolyzed for 15 minutes. (The use of more concentrated acid or a longer period of hydrolysis yielded identical results.) The hydrolyzed mixture was centrifuged and the clear supernatant used for analysis according to the method of Dische (11). The diphenylamine reagent was prepared by dissolving 1 gm. of diphenylamine (Merck's reagent) in 2 cc. of H<sub>2</sub>SO<sub>4</sub> and then adding 98 cc. of glacial acetic acid. The H<sub>2</sub>SO<sub>4</sub> was used

merely as a solvent for the diphenylamine. 8 cc. of this reagent were added to 4 cc. of the acid extract of the whole organism. The 12 cc. of solution were placed in a boiling water bath for 3 minutes and then rapidly cooled. After 5 minutes readings were made in an Evelyn photoelectric colorimeter with a No. 660 filter.

A curve had been plotted of varying known amounts of desoxyribose nucleic acid against corresponding Evelyn colorimeter readings. Therefore when we knew the colorimeter reading of an unknown solution, we could refer to the curve and determine the corresponding amount of desoxyribose nucleic acid in the unknown. Further acid extractions of the centrifuged hydrolyzed organisms gave negative qualitative tests for thymus nucleic acid.

All of these strains yielded from 2 to 6 per cent of the total dry weight of the bacteria as desoxyribose nucleic acid, which was about 10 to 30 per cent of the total nucleic acid. These results were not due to impurities in the broth, since organisms grown in a chemically defined medium, in which there was no nucleic acid, gave the same results as with the organisms grown in broth.

*Biological Properties*—The biological properties of each strain were studied at the time it was being analyzed chemically. A summary of these properties is given in Table III.

#### DISCUSSION

Fish sperm and tobacco ring spot virus have been shown to contain about 40 per cent nucleic acid (4). The data presented here show that the Group A  $\beta$ -hemolytic streptococci also contain considerable amounts of nucleic acid. Our findings further show that type-specific, virulent mucoid strains contain 19.3 to 22.5 per cent, less type-specific avirulent smooth strains 18.3 to 24.1 per cent, and non-type-specific avirulent rough strains 14.8 to 16.8 per cent nucleic acid. The rough colony form which is avirulent and non-type-specific thus shows a marked loss of 25 to 35 per cent nucleic acid, in contrast to the mucoid and smooth phases. Of the total nucleic acid contained in streptococci it appears that 10 to 30 per cent is desoxyribose (thymus) nucleic acid, the remainder being of the *d*-ribose (yeast) type. When our data are correlated with previously known facts, namely (a) occasional absence of virulence in the mucoid phase without change in colony form or type specificity and (b) the loss of virulence and

decrease or loss of type specificity in the smooth phase, it would appear that the above changes are not accompanied by any marked decrease in nucleic acid content. This also appears to be the case with respect to the nucleoprotein content.

In our study of the various streptococcal protein fractions we have always found nucleic acid. Heidelberger and Kendall (14) showed that all the protein fractions prepared from streptococcal cells contained phosphorus. Although these data might suggest that all the protein in streptococci is conjugated with nucleic acid in various proportions, one cannot as yet exclude the possibility of the existence of simple cellular proteins.

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# THE COUPLED OXIDATION OF CAROTENE AND FAT BY CAROTENE OXIDASE

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(Received for publication, April 12, 1940)

Recently Sumner and Dounce<sup>1</sup> described the enzyme discovered in 1928 by Bohn and Haas in legumes and especially in the soy bean. This enzyme peroxidizes the unsaturated fats and bleaches carotene, bixin, xanthophyll, and other carotenoids. We have called this enzyme "carotene oxidase," but possibly "unsaturated fat-peroxidizing enzyme" would be more accurate.

In earlier experiments the carotene we tested was a preparation dissolved in unsaturated oil. Later we employed crystalline carotene and were surprised to find that the carotene oxidase had an almost negligible bleaching action upon this. On the contrary, when one employs carotene dissolved in a small quantity of fat, the bleaching is extremely rapid. With excessive quantities of fat, the rate of bleaching of the carotene diminishes. Thus, the bleaching of 0.79 mg. of carotene required 18 to 20 hours with no oil, 32 seconds with 4.6 mg. of hemp-seed oil, and 180 seconds with 50 mg. of hemp-seed oil. A curve for the rate of the reaction is given in Fig. 1. Bixin, prepared from annatto paste, acts like carotene.

Using 4.6 mg. of hemp-seed oil and 0.79 mg. of carotene, we find the temperature optimum to be at 40–45°. This is quite different from the temperature optimum for the action of carotene oxidase upon cottonseed oil.

The effect of added fat upon the rate of bleaching of carotene is probably due to a coupled oxidation. Highly unsaturated fats, such as hemp-seed oil or soy bean oil, are much more effective,

<sup>1</sup> Sumner, J. B., and Dounce, A. L., *Enzymologia*, 7, 130 (1939). See also Hauge, S. M., *J. Biol. Chem.*, 108, 331 (1935) and Frey, C. N., Schultz, A. L., and Light, R. F., *Ind. and Eng. Chem.*, 28, 1254 (1936).

weight for weight, than olive oil or butter. Fat which has been previously peroxidized by the action of carotene oxidase has no increased action on the bleaching of carotene, nor does it cause immediate bleaching when carotene is dissolved in it, although bleaching occurs later. In order to exert its action, the fat must be mixed with the carotene before being suspended in water; if the fat and the carotene are suspended in water separately and then mixed, the action is slow.

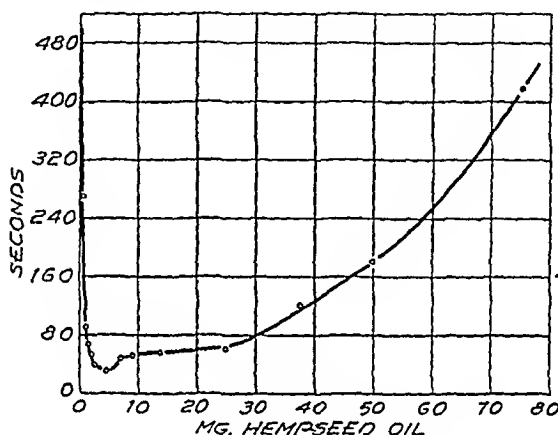


FIG. 1. The effect of hemp-seed fat upon the rate of oxidation of carotene by carotene oxidase.

#### EXPERIMENTAL

*Carotene Solution*—95 mg. of crystalline carotene (Nutritional Research Associates, South Whitley, Indiana) were shaken with 400 cc. of acetone and 200 cc. of 95 per cent alcohol until largely dissolved. The solution was then filtered and kept in a brown bottle.

*Fat Solution*—5 gm. of fat were dissolved in 200 cc. of acetone. The hemp-seed oil was prepared by grinding hemp-seed in a coffee mill, extracting with petroleum ether, filtering, and evaporating off the petroleum ether. The soy bean oil was prepared in a similar manner.

*Enzyme Solution*—5 gm. of fat-free soy bean meal were stirred with 200 cc. of distilled water and the suspension was centrifuged for  $\frac{1}{2}$  hour. The somewhat milky supernatant was poured off and kept in the ice chest.

*Phosphate Buffer*—This contained 4.73 per cent of  $\text{Na}_2\text{HPO}_4$  (anhydrous) and 4.54 per cent of  $\text{KH}_2\text{PO}_4$  (anhydrous). The pH was about 6.5.

*Procedure*—We pipetted into a 250 cc. Erlenmeyer flask 5 cc. of the carotene solution and added the fat solution to this if fat was to be employed. After mixing, we added 100 cc. of distilled water and 5 cc. of the phosphate buffer. Next 1 cc. of the enzyme was added and the contents of the flask were rotated until bleaching was judged to be complete. In experiments in which bleaching took over  $\frac{1}{2}$  hour, the rotation was intermittent.

#### SUMMARY

Carotene oxidase bleaches carotene with extreme slowness unless a small amount of fat is present. If an optimal quantity of fat is added, carotene is bleached with great rapidity. Bixin is affected in a similar manner. Highly unsaturated fats like hemp-seed oil or soy bean oil are more effective than olive oil or butter.

The bleaching action by the enzyme is probably an instance of a coupled reaction.



## THE OXIDATION OF VITAMIN E\*

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(Received for publication, April 1, 1940)

The rapid autoxidation of synthetic  $\alpha$ -tocopherol reported by Isler (1) is a demonstration on the pure substance of what has long been known regarding the behavior of vitamin E in the presence of readily autoxidizable fats (2). Some idea of what may happen to vitamin E under such conditions has been gained from a study of the effects of individual oxidizing agents, organic and inorganic, under controlled conditions.

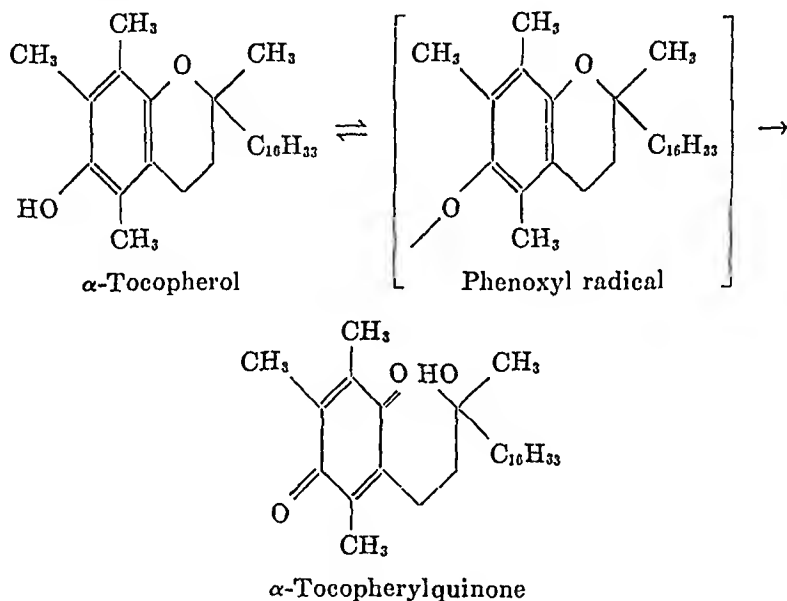
With gold chloride (3) or ferric chloride (4) the oxidation product is a substituted *p*-quinone which may be reduced to a tocopherylhydroquinone in neutral or weakly acid solutions. In the presence of halogen acids there is cyclization and regeneration of the original tocopherol. With more vigorous agents like silver nitrate or nitric acid the oxidation may proceed farther, to form a pyranobenzoquinone (5, 6); this compound has been shown to be devoid of biological activity. The biological activity of the *p*-quinone produced by ferric chloride or by mild treatment with silver nitrate (7) is still open to question; ferric chloride at steam bath temperature (4, 8) produced an inactive substance, whereas the product obtained at room temperature (9) was still effective as vitamin E.

The change from tocopherol to tocopherylquinone might occur in two successive univalent steps; such a two-step oxidation would be in accordance with Michaelis' theory of compulsory univalent oxidation (10). According to Conant (11) and Fieser (12), in the irreversible oxidation of a phenolic substance, the initial step is reversible and consists of the production of a phenoxyl radical

\* A report of this work was presented by one of us (C. G.) before the meeting of the American Society of Biological Chemists at New Orleans, March 13-16, 1940.

which forms an unstable oxidation-reduction system with the phenol. When such a reversible step governs the rate of the irreversible oxidation of a phenol, it is possible to measure an apparent oxidation potential by the method of potentiometric indicators.

Such a system may also be postulated between  $\alpha$ -tocopherol and  $\alpha$ -tocopherylquinone as follows:



Successful measurement of an apparent oxidation potential of  $\alpha$ -tocopherol would in itself be suggestive evidence for the existence of the system and for the irreversibility of the second step in it. The purpose of this paper is to report the apparent oxidation potential of synthetic  $\alpha$ -tocopherol and to record the biological inactivity of  $\alpha$ -tocopherylquinone.

#### EXPERIMENTAL

The procedure of Conant and Pratt (13) was applied to  $\alpha$ -tocopherol at a temperature of 75° in alcoholic solution (Table I). Under the conditions of the experiment, the apparent oxidation potential, *i.e.* the potential at which 20 to 30 per cent of the  $\alpha$ -tocopherol was oxidized in 30 minutes, lies between the normal potentials of a mono- and a dimethylhydroquinone. Duplicate

experiments agreed within a few millivolts. The same range was obtained when the procedure of Conant and Pratt was modified; the proportion of equimolar mixtures of the reagent quinones and corresponding hydroquinones to  $\alpha$ -tocopherol was increased and

TABLE I

*Oxidation of  $\alpha$ -Tocopherol in 95 Per Cent Alcohol (0.2 N in HCl) at  $75^\circ \pm 2^\circ$*

Reagent (containing equimolar amounts of reduced form)*	$E_{\text{st}}$	$\alpha$ -Tocopherol†					
		Change of potential			Fraction changed		
		5 min.	15 min.	30 min.	5 min.	15 min.	30 min.
	volt	millivolts	millivolts	millivolts	per cent	per cent	per cent
Quinone.....	0.711	-92	-112	-119	>95	>95	>95
Toluquinone.....	0.656	-15	-22	-28	22	63	73
p-Xyloquinone.....	0.597	-1	+1	0	0	0	0
$\psi$ -Cumoquinone.....	0.537§	+1	+1	0	0	0	0
2-Methyl-1,4-naphthoquinone.....	0.408	+1	0	0	0	0	0

\* The concentration of both the oxidized and reduced forms was  $5 \times 10^{-4}$  M.

† The normal oxidation-reduction potentials are not corrected for pH or temperature.

‡ The concentration of  $\alpha$ -tocopherol was always  $1 \times 10^{-3}$  M.

§ Calculated.

TABLE II

*Oxidation of  $\alpha$ -Tocopherol by Quinones in 95 Per Cent Alcohol (0.02 N in Acetic Acid and Sodium Acetate) at  $75^\circ \pm 2^\circ$*

Reagent*	$E_{\text{st}}$	$\alpha$ -Tocopherol		
		Amount used‡	Amount recovered	Amount oxidized in 30 min.
	volt	mg.	mg.	per cent
Quinone.....	0.711	10.7	1.47	86
Toluquinone.....	0.656	10.0	6.2	38
p-Xyloquinone.....	0.597	9.6	8.25	14
Trimethylquinone....	0.537§	9.8	9.13	7
None.....		4.4	4.4	0

\* The reagent in all cases was  $2 \times 10^{-4}$  M in the oxidized and in the reduced form.

† These potentials are not corrected for temperature and pH.

‡ In all cases the concentration was  $2 \times 10^{-5}$  M.

§ Calculated.



the extent of oxidation of  $\alpha$ -tocopherol in 30 minutes was determined by gold chloride titration of the unchanged tocopherol (Table II). From the figures in the last column it is apparent that under the conditions the amount of  $\alpha$ -tocopherol oxidized decreased with decreasing oxidation-reduction potential of the reagent mixtures and that the apparent oxidation potential

TABLE III

*Vitamin E Action of  $\alpha$ -Tocopherylquinone and  $\alpha$ -Tocopherylhydroquinone*

Compound	Amount fed	No. of animals	No. of implantations	No. of resorptions	No. of litters
	mg.				
$\alpha$ -Tocopherylquinone	5	1	1	1	0
	15	2	2	2	0
	35	1	1	1	0
$\alpha$ -Tocopherylhydroquinone*	15	2	2	2	0
“ triacetate†	15	3	3	3	0
$\alpha$ -Tocopherol acetate	2.7	9	9	1	8
No dosage		4	4	4	0
Olive oil	200	5	5	5	0
<i>p</i> -Xyloquinone	50	2	2	2	0
“ +	50	2	2	2	0
Phytol	120				
<i>p</i> -Xylohydroquinone	50	2	2	2	0
“ +	50	2	2	2	0
Phytol	120				
<i>p</i> -Xylohydroquinone +	50	2	2	2	0
Phytadiene	150				
Tolhydroquinone +	50	2	2	2	0
Phytol	120-170				

\* Administered intraperitoneally.

† Not recrystallized.

(20 to 30 per cent oxidation) of  $\alpha$ -tocopherol lies between the normal potentials of toluquinone and *p*-xyloquinone.

The biological tests of  $\alpha$ -tocopherylquinone were made on virgin animals reared on a vitamin E-deficient diet. The quinone was prepared from synthetic  $\alpha$ -tocopherol<sup>1</sup> by ferric chloride or gold

<sup>1</sup> The synthetic  $\alpha$ -tocopherol was prepared by alkaline hydrolysis of  $\alpha$ -tocopheryl acetate which was kindly furnished by Hoffmann-La Roche, Inc.

chloride oxidation according to the procedures of John (4) and Karrer (3), respectively.  $\alpha$ -Tocopherylquinone, its hydroquinone, and the hydroquinone triacetate displayed no vitamin E activity even at the high levels fed (Table III). Eight of the nine control animals given 2.7 mg. of  $\alpha$ -tocopherol acetate gave a positive response; nine animals, serving as negative controls, showed that we were not dealing with first litter fertility. Subsequently, five of the nine animals used for assaying the derivatives of  $\alpha$ -tocopherol were given adequate doses of vitamin E and all five had litters.

TABLE IV

*Antioxygenic Action of  $\alpha$ -Tocopherol and  $\alpha$ -Tocopherylquinone in Lard at 75°*

Substance	$\alpha$ -Tocopherol content*	Antioxygenic index†	Concentration
	<i>per cent</i>		<i>per cent</i>
I. $\alpha$ -Tocopherylquinone	0*	1	0.10
II. "	4*	1	0.10
III. "	20*	2	0.03
IV. "	50†	2.5	0.04
V. $\alpha$ -Tocopherol	98*	2-3	0.02
VI. "	98-100§	5	0.04

\* As determined by electrometric titration against gold chloride (Karrer).

† Ratio of the induction period of stabilized fat to that of the control.

‡ A mixture of Substances I and V.

§ Substance VI was assayed against Substance V colorimetrically (Emmerie and Engel (16)).

*p*-Xyloquinone, *p*-xylohydroquinone, and toluhydroquinone which form oxidation-reduction systems in the same potential range as  $\alpha$ -tocopherol had no vitamin E activity (Table III). Mixtures of these compounds with phytol and phytadiene were likewise ineffective. This same demonstration has already been made with trimethylhydroquinone and phytol by Evans and collaborators (14). Apparently, the rat lacks the requisite catalysts for cyclizing any of these compounds to the chroman structure.

Oxidation of  $\alpha$ -tocopherol by ferric chloride at room temperature is incomplete. We have used the accurate electrometric titration

method of Karrer (3) to detect the presence of  $\alpha$ -tocopherol in samples of  $\alpha$ -tocopherylquinone prepared by different methods.<sup>2</sup> At room temperature 20 per cent of the  $\alpha$ -tocopherol remained unattacked, whereas nearly complete oxidation (96 per cent) was secured at 75°. The antioxidant properties of  $\alpha$ -tocopherol were also used to detect its presence in  $\alpha$ -tocopherylquinone preparations.  $\alpha$ -Tocopherol is an antioxidant for lard (15);  $\alpha$ -tocopherylquinone is not. Tocopherylquinone preparations containing 20 per cent or more  $\alpha$ -tocopherol (Table IV) displayed antioxygenic properties but preparations containing 4 per cent or less of tocopherol (determined by electrometric titration) were not inhibitors at the same or even at higher concentrations.

### DISCUSSION

The suggestion has been advanced provisionally by John and coworkers (4) and also by Evans and associates (14) that the biological activity of tocopherols may be related to their ready transformation *in vitro* to quinones, in a reversible oxidation-reduction system. If such a system extended from  $\alpha$ -tocopherol to  $\alpha$ -tocopherylquinone, the latter as well as the former should have biological activity. Since  $\alpha$ -tocopherylquinone appears not to have biological activity, we must conclude that if an oxidation-reduction system is concerned it must be between tocopherol and an intermediate phenoxyl radical. The significance of these observations in relation to the physiology of vitamin E is under investigation.

Our thanks are due to Lever Brothers Company of Cambridge, Massachusetts, for a grant in support of this work.

### SUMMARY

1. An apparent oxidation potential of  $\alpha$ -tocopherol has been measured by two methods; it appears to be between the normal oxidation potentials of mono- and dimethylhydroquinones.
2. Even in relatively large doses  $\alpha$ -tocopherylquinone does

<sup>2</sup> Emerson, Emerson, and Evans (9) claimed that the development of color with Folin and Ciocalteu's phenol reagent is a sensitive test for  $\alpha$ -tocopherol in tocoquinone preparations. In our hands, this color test has proved unreliable, since quinones respond positively and in a shorter time than has been stated.

not possess biological activity as measured by the capacity to cure sterility in female rats reared on a vitamin E-deficient diet. Contrary reports which have appeared may be explained by the presence of unoxidized  $\alpha$ -tocopherol. Unless ferric chloride is used at steam bath temperature, oxidation is incomplete. With gold chloride, oxidation is complete at room temperature.

3.  $\alpha$ -Tocopherylquinone containing unoxidized  $\alpha$ -tocopherol demonstrates antioxygenic action on lard, whereas  $\alpha$ -tocopherylquinone itself has no such action.

4. Various substituted hydroquinones, having oxidation potentials in the same range as  $\alpha$ -tocopherol, are inactive biologically either alone or in association with phytol.

5. These observations suggest that the reaction  $\alpha$ -tocopherol  $\rightarrow$   $\alpha$ -tocopherylquinone is not reversible in the organism. If, as is probable, the reaction takes place in two steps, the first step is reversible and may have biological significance.

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# THE ADSORPTION OF FLUORIDES BY ENAMEL, DENTIN, BONE, AND HYDROXYAPATITE AS SHOWN BY THE RADIOACTIVE ISOTOPE\*

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Evidence has recently appeared from three diverse sources that fluorine is in some way related to the inhibition of dental caries. First, chemical analyses have shown that sound enamel from sound teeth contains more fluorine than that from carious teeth (1); this is the only established chemical difference between sound and carious tissues. Second, field studies have shown a lower incidence of caries in areas of mottled enamel (dental fluorosis (2,3)). Third, in animal experimentation, rats have been found to develop less caries on a caries-producing diet when fluorine is added to the diet (4, 5). Two factors in the inhibiting action of fluorine have been discovered: (a) fluorine reduces the acid production of oral bacteria (6) and (b) fluorine reduces the solubility of the dental tissues (7).

Since human dental caries begins on the surface of the enamel, it is important to explore the possibility that fluorides are adsorbed on the surface. To investigate this problem, samples of powdered enamel, dentin, bone, and hydroxyapatite were exposed to solutions of sodium fluoride. The amount of fluorine picked up by these calcium phosphates was found to follow the adsorption isotherm, thus demonstrating that fluorine does adsorb on dental tissues.

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## EXPERIMENTAL

Radioactive fluorine was prepared by bombarding oxygen in water molecules by the proton beam of the cyclotron. The nuclear reaction is as follows:  ${}_8\text{O}^{18} + {}_1\text{H}^1 \rightarrow {}_9\text{F}^{18} + {}_0n^1$ .

The unstable isotope  $\text{F}^{18}$  has a half life of 112 minutes and reverts to  $\text{O}^{18}$  by the emission of a positron as follows:  ${}_9\text{F}^{18} \rightarrow {}_8\text{O}^{18} + {}_{+1}e^0$ . Approximately 4 cc. samples of distilled water were bombarded for 2 hours in a special water-cooled cell furnished with a thin aluminum window (thickness 0.001 inch) through which the proton beam passed. Each of these samples was made up to 10 cc. with distilled water; 1 cc. was taken, diluted to 10 cc., and used as a standard for obtaining the decay curve. Suitable aliquots of the radioactive fluorine solutions were made up to volumes of 50 cc. with solutions of sodium fluoride varying in concentration from 1:100 to 1:1,000,000 by powers of 10. The enamel and dentin samples were prepared by the centrifugal flotation method (8) from dried teeth; the samples of bone were from inorganic residues prepared by KOH-ethylene glycol extraction (9). All the tissue samples were powdered to pass a 60 mesh screen. The hydroxyapatite was sample "TR 5" (Ca:P ratio, 2.12) described by Hodge, LeFevre, and Bale (10). It was not screened.

25 cc. of the sodium fluoride solutions containing radioactive fluorine were stirred with 50 mg. samples of enamel, dentin, bone, or hydroxyapatite for 30 minutes at  $40^\circ$ . The solutions were decanted after centrifugation, and the precipitates twice washed with distilled water. The precipitates were dissolved in approximately 2 cc. of 6 N hydrochloric acid; solutions of dentin samples were centrifuged to remove the insoluble flakes of organic matter. 2 cc. samples of these solutions were placed in the counting cup of the Geiger-Müller scale-of-four counter (11), and the number of counts determined, for duplicate or triplicate 5 minute periods. At intervals of 3 to 4 hours, the radioactivity of the standard radioactive fluorine solutions was determined as well as the background count and the counter sensitivity (11). It was shown experimentally that the variations in the concentration of calcium phosphate from solution to solution produced negligible changes in  $\beta$ -ray absorption.

The decay curves of radioactive fluorine were plotted for each experiment and from these curves the counts for the various samples of dissolved calcium phosphates were calculated back to the 100 per cent basis. The mg. of fluorine picked up by each calcium phosphate sample were calculated, and from these data two values were obtained (1) the mg. of fluorine picked up per gm. of calcium phosphate, and (2) the concentrations of fluorine remaining in the solutions at equilibrium. These values were plotted logarithmically with the latter as ordinates (Fig. 1). In the calculations the amounts of radioactive fluorine are so mi-

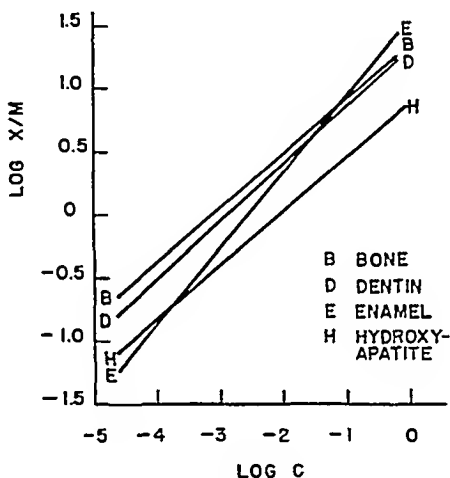


FIG. 1. Adsorption isotherms (40°) for the various calcium phosphates

nute that the total fluorine concentrations are taken as those of the  $F^{19}$  isotope. The straight lines given in Fig. 1 were calculated by the method of least squares; the coefficients obtained for the equation of each calcium phosphate were then expressed in terms of the coefficients  $n$  and  $k$  from the Freundlich adsorption isotherm equation.

### Data

In Table I are given the averages for the mg. of fluoride adsorbed per gm. of adsorbent for each of the five dilutions of fluoride used; each figure represents the average of four to six



determinations. The percentages of the total dissolved fluoride which are adsorbed increase from 0.5 per cent at 1:100 NaF to 1, 5, 20, and 40 per cent, respectively, as the fluoride concentration decreases by successive powers of 10. Since the values for the logarithms of  $x/m$  when plotted against the values of logarithms of  $C$  for enamel, dentin, bone, and hydroxyapatite in each case fall along a straight line (Fig. 1), it is evident that this criterion of the Freundlich adsorption isotherm is satisfied and that these substances adsorb fluorine.

In the following tabulation the adsorption coefficients for fluorine on the various calcium phosphates are shown. The coefficient  $n$ , characteristic of the adsorbed substance for adsorbents of

TABLE I  
*Fluoride Adsorbed on Various Calcium Phosphates at Various Fluoride Dilutions*

Concentration of F in solution at equilibrium, $C$	F adsorbed per gm. adsorbent ( $x/m$ )			
	Enamel	Dentin	Bone	Hydroxyapatite
$x \times 10^3$	mg.	mg.	mg.	mg.
230	12	13	12	2.7
23	4.3	2.5	2.8	1.2
2.2	1.0	1.2	1.4	0.65
0.18	0.32	1.23	0.49	0.34
0.02	0.08	0.29	0.07	0.05

identical chemical composition, should be reasonably constant for each of these calcium phosphates, since they have apparently identical principal molecular lattices (12). The  $n$  values given are considered to be in satisfactory agreement although the value for enamel is somewhat lower than the values for the other substances.  $k$ , the coefficient characteristic of the adsorbing substance, is different for various calcium phosphates.

Symbol	Enamel	Dentin	Bone	Hydroxyapatite
$n$	1.6	2.2	2.1	2.3
$k$	37	22	22	8

A few experiments were done in which crowns of teeth were dipped for 30 minutes at 40° into solutions of sodium fluoride containing radioactive fluorine. The enamel was partially dis-

solved off by dipping the tooth into a solution of hydrochloric acid. Small, but detectable amounts of fluorine were adsorbed by these intact enamel surfaces; the maximum amount of fluorine adsorbed on a single tooth was of the order of 0.02 mg.

#### DISCUSSION

It would be almost meaningless to attempt to differentiate between fluoride adsorption as a physical phenomenon and as a chemical reaction, since there is an excellent chance that fluoride approaching the surface of a hydroxyapatite particle would react to produce fluorapatite (13-15), a mixed apatite, or calcium fluoride (16). In fact, the evidence of a surface reaction may have been an artifact contributed by the relatively short exposure of the calcium phosphates to the fluoride solutions (17).

The  $n$  values characteristic of the adsorbed substance are all of the same order. These values are so nearly identical in the case of bone, dentin, and hydroxyapatite that these curves (Fig. 1) might be described as affine adsorption curves. The curve for enamel crosses the other three curves; it is lower at low fluoride concentration than that of hydroxyapatite and higher in high concentration than those of bone or dentin. The significance of this deviation is unknown. It is interesting to note that the  $n$  values obtained by Manly and Levy (18) for the adsorption of phosphates on enamel, dentin, and bone are of the same numerical order as those for fluorine. For both fluorine and phosphate the  $n$  values for dentin and bone are similar; for enamel the  $n$  value for phosphate is higher, whereas for fluorine it is lower than the dentin and bone values.

The  $k$  values characteristic of the adsorbing substance are large for enamel (37), medium for dentin and bone (22), and small for hydroxyapatite (8). The higher value for enamel rules out any pronounced influence on fluoride adsorption due to the fluoride already present, since dentin contains normally about twice as much fluoride as enamel. The  $k$  fluorine to  $k$  phosphate ratio is 26 for enamel and approximately 3 for dentin and bone. The reason for this is unknown. However, such differences in ratios are not unknown; in fact Freundlich states, "Undoubted regularities holding for the true adsorptive power are neither numerous nor well established" (19).

The fact of adsorption of fluorides by enamel may have clin-

ical significance. The local application of solutions of fluorides to teeth *in situ* might add sufficient fluorine to the enamel surface to decrease its susceptibility to dental caries.

#### SUMMARY

1. Enamel, bone, dentin, and hydroxyapatite are shown to adsorb fluorine according to the Freundlich adsorption isotherm.

2. The coefficients characteristic of the adsorbed substance ( $n$ ) and of the adsorbent ( $k$ ) have been calculated for each substance.

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*Correction*—The coefficients  $a$  and  $b$  in the equation  $y = a + bx$  (Fig. 1) should be respectively, dentin, 0.64, 0.50; bone, 1.20, 0.51; hydroxyapatite, 1.17, 0.42. This does not alter the major conclusions.

## THE METABOLISM OF *d*-SORBITOL

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*d*-Sorbitol has been employed in Europe as a carbohydrate substitute in the treatment of diabetes and its use for this purpose has led to a highly controversial literature on the subject. There are those who maintain that it is not metabolized (1) and may be safely used as a sugar substitute (2-4) in the diabetic diet, while others (5, 6) claim it produces hyperglycemia and should be avoided (7-10). The variety of results reported as to the metabolism and glycogenic ability of sorbitol in the fasting animal has contributed to this controversy. According to Payne, Lawrence, and McCance (1) sorbitol fails to increase liver glycogen in the rat, while Todd, Myers, and West (11) report considerable deposition of liver glycogen after it was fed or injected. Waters (12) claims that the feeding of sorbitol solution to fasted rats produced no increase in hepatic glycogen, but that intra-peritoneal injection resulted in a marked increase in the glycogen content of the liver. Carr and Forman (13) find this hexahydric alcohol to be glycogenic when fed with two-thirds cacao butter to rats. Lafon (14) reports that sorbitol, up to 30 per cent of the diet, is not toxic to mice and seems to be almost completely utilized. Roche and Raybaud (10) state that sorbitol is not converted into liver glycogen in the rabbit and that it does not prevent insulin hypoglycemia. Thannhauser and Meyer (15) find that when fed with lean meat to dogs this alcohol can be converted into liver glycogen as readily as glucose.

Much of the controversy as to the utilization of sorbitol by the fasted animal appears to be due to the facts that conclusions have been based mainly on experiments performed with very small

series of animals and that experimental conditions have varied greatly. The present study deals with the metabolism of sorbitol in a number of rats which had fasted for different periods. Various amounts of sorbitol were given and the effects of 3 and of 6 hour absorption periods were studied. The question of the metabolism of sorbitol has become increasingly important, as in recent years its price has been greatly reduced and its use extended. It has already been incorporated in bread as a moistening agent and is used in the manufacture of candy. The question naturally arises as to whether or not this polyhydric alcohol can be classed as a foodstuff.

#### EXPERIMENTAL

An approximately equal number of normal male and female white rats (aged 92 to 131 days) were used for each experiment. In the first group, the animals were fasted for 24 hours and then given 2 cc. of a 25 per cent solution of sorbitol which had been recrystallized from pyridine according to the method of Strain (16). We shall designate this as Sorbitol I. In the second group of experiments, food was withheld from the rats for 46 hours; then they were given either 2 cc. of a 25 per cent solution of Sorbitol I or Sorbitol II, a highly purified product of Atlas.<sup>1</sup> 3 hours later an additional 500 mg. of Sorbitol I or II were given. After another 3 hour absorption period, the animals were anesthetized with sodium amytal and their hind legs skinned. The legs and liver were then frozen *in situ* with CO<sub>2</sub> snow. The methods for the preparation of the tissues for analysis and for the determination of tissue carbohydrates have been described (17).

In Table I are listed the glycogen, lactic acid, and fermentable and non-fermentable sugar content of the various tissues examined for the experimental animals fasted 24 hours and given 500 mg. of Sorbitol I together with the control values. The values for the control rats are those obtained over a period of 4 years in separate experiments dealing with carbohydrate metabolism. The observations were sufficiently uniform to justify combining them. As the hepatic glycogen of males is greater than that of females, we have separated the liver glycogen values

<sup>1</sup> The sorbitol was generously furnished by the Atlas Powder Company.

TABLE I

*Carbohydrate Content of Tissues of Control and Experimental Rats*

The results are expressed in mg. per 100 gm. except for blood, in which case they are recorded as mg. per 100 cc. The values for glycogen are given in terms of glucose.

Figures in bold-faced type represent values for the rats given Sorbitol I.

Substance determined	Maximal	Minimal	Mean	Standard deviation of mean	No. of rats	
					Males	Females
Glycogen, liver.....	603	26	203	28		30
" " .....	1000	97	317	30		9
" " .....	1847	94	535	67	41	
" " .....	723	233	474	11	9	
" muscle.....	749	370	563	11	40	29
" " .....	607	412	535	12	9	9
Lactic acid, liver.....	17.5	5.5	10.0	0.5	23	14
" " " .....	10.9	6.2	8.0*	0.4	9	6
" " muscle.....	54.2	11.8	22.8	1.2	31	20
" " " .....	30.2	12.2	21.3	1.5	9	5
" " blood.....	21.8	8.1	13.8	1.0	8	8
" " " .....	25.7	7.6	12.6	1.8	5	6
Fermentable reducing substances						
Liver.....	220	51	104	4.1	34	19
" .....	148	38	87*	6.3	9	9
Muscle.....	42	4	17	1.0	36	19
" .....	49	7	20	2.5	9	9
Kidney.....	82	15	53	2.0	35	19
" .....	102	15	47	4.0	9	9
Blood.....	92	59	74	1.7	11	12
" .....	84	66	76	2.1	5	5
Non-fermentable reducing substances						
Liver.....	59	0	26	1.9	34	19
" .....	68	13	31	3.5	9	9
Muscle.....	45	4	17	1.2	36	19
" .....	35	4	14	1.6	9	9
Kidney.....	37	5	17	1.1	35	19
" .....	43	9	19	2.1	9	9
Blood.....	8	2	5	0.4	11	12
" .....	4	2	2*	0.4	5	5

\* Statistically significant by the *t* test of Fisher.

according to the sex of the animal. In the case of muscle glycogen there appears to be no sex difference and we have therefore made no distinction between males (mean 566 mg.) and females (mean

TABLE II

*Carbohydrate Content of Tissues of Control and Experimental Rats*

The results are expressed in mg. per 100 gm. except for blood, in which case they are recorded as mg. per 100 cc. The values for glycogen are given in terms of glucose.

Substance determined	Type of sorbitol fed	Maximal	Minimal	Mean	Standard deviation of mean	No. of rats	
						Males	Females
Glycogen, liver.....		378	54	165	32		10
“ “ .....	I	118	0	46*	14		10
“ “ .....	II	376	0	126	43		10
“ “ .....		786	67	371	70	10	
“ “ .....	I	1168	256	784*	87	10	
“ “ .....	II	1166	229	627*	104	10	
“ muscle.....		626	300	501	18	10	10
“ “ .....	I	672	417	536	15	10	10
“ “ .....	II	722	349	540	23	10	10
Lactic acid, liver.....		12.2	5.4	7.6	0.4	10	10
“ “ “ .....	I	14.7	5.8	8.8*	0.4	10	10
“ “ “ .....	II	12.9	6.1	9.8*	0.5	10	10
“ “ muscle.....		38.6	9.8	22.8	2.0	10	9
“ “ “ .....	I	32.8	13.2	24.1	1.2	10	10
“ “ “ .....	II	33.7	10.1	20.8	1.6	10	10
“ “ blood.....		14.0	6.6	10.0	0.7	5	5
“ “ “ .....	I	19.6	8.2	12.2	1.0	5	5
“ “ “ .....	II	14.3	7.9	10.5	0.7	5	5
Fermentable reducing substances							
Liver.....		191	40	104	8.4	10	10
“ .....	I	143	62	104	1.4	10	10
“ .....	II	174	74	110	5.9	10	10
Muscle.....		28	10	20	1.3	10	10
“ .....	I	30	10	20	1.5	10	10
“ .....	II	31	11	19	1.2	10	10
Kidney.....		69	23	48	2.8	10	10
“ .....	I	81	17	54	3.3	10	10
“ .....	II	125	31	69*	5.4	10	10
Blood.....		78	66	72	1.4	5	5
“ .....	I	89	67	77	2.3	5	5
“ .....	II	89	67	78*	1.8	5	5

TABLE II—*Concluded*

Substance determined	Type of sorbitol fed	Maximal	Minimal	Mean	Standard deviation of mean	No. of rats	
						Males	Females
Non-fermentable reducing substances							
Liver.....		58	11	29	3.2	10	10
“ .....	I	45	10	29	2.4	10	10
“ .....	II	41	8	20*	1.5	10	10
Muscle.....		31	6	14	1.5	10	10
“ .....	I	25	7	15	1.1	10	10
“ .....	II	24	6	14	0.4	10	10
Kidney.....		47	7	23	2.7	10	10
“ .....	I	30	7	17*	1.6	10	10
“ .....	II	47	7	21	2.3	10	10
Blood.....		6	2	4	0.5	5	5
“ .....	I	3	2	2*	0.1	5	5
“ .....	II	3	2	2*	0.2	5	5

\* Statistically significant by the *t* test of Fisher.

559 mg.). The maximal, minimal, and mean values for the carbohydrate content of the various tissues are given together with the standard deviation of the mean. The means which are statistically significant by the *t* test of Fisher (18) are indicated. It is evident that sorbitol is not metabolized under the above conditions. Those means which differ statistically from controls represent decreases and are not readily interpreted.

Values for rats fasted for a longer period and in which the sorbitol dose was divided are summarized in Table II. These rats were fasted for 46 hours and then given two doses of 500 mg. of sorbitol at 3 hour intervals. The controls were fasted for 52 hours. It will be seen that there is no increase in the liver glycogen of females, a significant decrease being noted in the case of Sorbitol I. There is a significant increase in liver glycogen of males but in one instance this is on the border line of significance. Increases are noted for liver lactic acid and fermentable reducing substances of the blood. As the total carbohydrate for the blood is about the same for experimental and control values, it is doubtful whether or not the slight rise in fermentable sugar is of any



significance. Decreases in the non-fermentable sugar of the blood may be due to differences of the various yeasts employed.

A comparison of Table I with Table II shows that sorbitol utilization is apparently favored by the giving of large doses in divided portions and by allowing a longer period of absorption. This may be accounted for by the fact that sorbitol is very poorly absorbed from the gastrointestinal tract. Practically all of the animals given the alcohol developed severe diarrhea. Unfortunately, we were not able to determine the coefficient of absorption for sorbitol. Considerable time was spent in an endeavor to adapt the Silberstein, Rappaport, and Reifer (19) method for the determination of sorbitol in blood to the gastrointestinal extract but without success. We were likewise unable to use this method for blood because of the unreliable results obtained.

#### DISCUSSION

Our data give no indication that sorbitol is utilized when given after the customary fasting period of 24 hours. It is also questionable whether utilization occurs when the animals are fasted for 48 hours and are then given large quantities of the alcohol over a longer period of time. Under the latter conditions, male rats appeared to store glycogen in their livers but the females did not. It is difficult to reconcile our findings with those of Todd and coworkers who observed glycogen formation under approximately the same conditions. It should be pointed out, however, that they cite only three experiments in which sorbitol was given orally. These authors do not state the sex of their rats. This is important, as it is not uncommon to find values of 1 per cent or more glycogen in the liver of male rats fasted for 24 hours. Todd and his associates decapitated the animals before removal of the liver, and then ground the liver in a meat grinder. By our method of freezing the living tissues before removal, and powdering them while frozen, we should have obtained the higher glycogen values.

There can be no question but that sorbitol forms glycogen when fed mixed with cacao butter, as shown by Carr and Forman and by Todd and his coworkers. Cacao butter is known to lessen peristalsis, and when incorporated with sorbitol apparently prevents the diarrhea which is almost invariably produced by in-

gestion of aqueous solutions of the alcohol. The inability to show glycogen formation after the oral administration of sorbitol is probably related to the low coefficient of absorption of the alcohol. Unmistakable formation of glycogen occurs when sorbitol is given intraperitoneally (11, 12).

The increased lactic acid content of the liver after sorbitol administration is interesting in view of the fact that we have found that the feeding of fructose gives a like result.<sup>2</sup> It has been postulated by others (12, 20, 21) that sorbitol first may be changed to fructose before undergoing further metabolism. This oxidation evidently takes place in the liver. When sorbitol was given to fructosuric individuals, the blood fructose reached a level of 35 mg. per cent, and from 6.4 to 12 per cent of the sorbitol appeared in the urine as fructose (20, 21). These latter observations afford strong evidence for the utilization of sorbitol by man.

#### SUMMARY

Glycogen formation was not observed when sorbitol was fed to rats after the customary fasting period of 24 hours. It is questionable whether utilization occurred when the animals fasted 46 hours and were then given larger quantities of the alcohol over a longer period of time. The inability to form glycogen under these circumstances is probably dependent upon the low rate of absorption of sorbitol from the gastrointestinal tract.

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<sup>2</sup> Unpublished experiments.

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## THE DECOMPOSITION OF SELENIFEROUS PROTEINS IN ALKALINE SOLUTIONS\*

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The selenium in cereal grains, which Franke and Painter (1936) found to be confined mostly to the proteins, is in fairly stable combination and acid hydrolysis of such proteins yields organic selenium compounds. Westfall and Smith (1939) seem to question the conclusions of Painter and Franke (1935, 1936) and Horn, Nelson, and Jones (1936) regarding the nature of selenium in cereals. They extracted cereals and proteins with oxidizing agents, then distilled the extracts with a bromine-hydrobromic acid solution, and were able to reduce selenium in the distillates to the elemental form. Although this is a general property of inorganic selenium, their results can also be explained by reactions of many organic selenium compounds. The oxidizing agents used by Westfall and Smith readily convert the selenium of many organic selenium compounds to a form easily cleaved. Diselenides give seleninic acids and Painter (1939) has found these compounds to be cleaved in acidic as well as in basic solutions to give inorganic selenite. Some selenium ethers likewise yield elemental selenium upon treatment with oxidizing agents followed by reduction. The selenium in plants and known organic selenium compounds has not been reduced to the metallic form without the use of reagents which first change organic selenium to inorganic selenite or selenate.

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Owing to the chemical properties of selenium, its occurrence in the protein, and the properties of the selenium compounds in the hydrolysates, it has been suggested by Painter and Franke (1935) that selenium analogues of the sulfur amino acids may exist in nature. Although the deposition of selenium follows closely that of sulfur in cereals and cereal proteins, this is not true for all plants (Painter and Franke, 1940). Inorganic sulfates

TABLE I  
*Selenium Content of Alkali Hydrolysates*

Hydrolysate No.	Material hydrolyzed	Selenium before alkaline hydrolysis	Method of hydrolysis	Selenium in soluble hydrolysate
		p.p.m.		p.p.m.
1	Wheat Gluten 582	117	51 hrs. in 14% Ba(OH) <sub>2</sub> ·8H <sub>2</sub> O	85
2	" " 582	117	51 " " 14% " + PbO + SnCl <sub>2</sub>	40
3	" " 582	117	56 hrs. in 6.5% Ca(OH) <sub>2</sub>	99
4	" " 582	117	56 " " 6.5% " + PbO	50
5	Corn Protein 523	143	51 " " 14% Ba(OH) <sub>2</sub> ·8H <sub>2</sub> O + PbO + SnCl <sub>2</sub>	42
6	Acid hydrolysate of Gluten 582*	95	19 hrs. in 14% Ba(OH) <sub>2</sub> ·8H <sub>2</sub> O + PbO + SnCl <sub>2</sub>	50
7	" " *	95	19 hrs. in 6.5% Ca(OH) <sub>2</sub>	70
8	" " †		1 hr. in 14% Ba(OH) <sub>2</sub> ·8H <sub>2</sub> O + PbO	7

\* Hydrolyzed by boiling 14 hours in approximately 33 per cent H<sub>2</sub>SO<sub>4</sub> (see Painter and Franke (1936)).

† Hydrolyzed by boiling 5 hours in 18 N H<sub>2</sub>SO<sub>4</sub> (see Painter and Franke (1936)).

can readily be detected in plants but the authors have been unable to demonstrate the presence of inorganic selenates or selenites in plants.

Franke and Painter (1936) showed that some of the selenium was alkali-labile when they electrolyzed a seleniferous gluten peptized in dilute alkali. A small amount of selenium was detected in the anodic cell. Soon after this, attempts were made to remove all the selenium from the protein by hydrolysis in alkali-

line plumbite. The lead sulfide, which always contained selenium and other insoluble material, was filtered out and the hydrolysates dried. The selenium content of these hydrolysates is shown in Table I.

Some of the hydrolysates in Table I contained a small amount of alkali, but the reduction of the selenium content was greater than can be attributed to the increase in weight over the protein by the water added by hydrolysis and unremoved alkali. It can be seen that there was a greater reduction of the selenium content when plumbite was present during hydrolysis.

Judging from the ease with which selenium can be removed from the protein when it is hydrolyzed in alkaline solution, additional evidence is at hand to indicate that selenium may take a place in the molecule analogous to sulfur. It is generally assumed that all alkali-labile sulfur in proteins arises from cystine or cysteine. Andrews (1928), Gortner and Sinclair (1929), Thor and Gortner (1932-33), Clarke and Inouye (1930), and others have shown that the quantity of sulfur removed from cystine depends upon the method of hydrolysis. Labile sulfur values are reported for only a few proteins. It was therefore decided to hydrolyze sufficient protein material in different alkaline solutions to permit the estimation of both selenium and sulfur in the same hydrolysate fractions so that their relative stability could be compared.

The results in Table II show that the stability of selenium in proteins is comparable to that of sulfur, but in every case a greater proportion of selenium than of sulfur remained in the filtrate from the lead precipitate.

It is assumed that by the alkaline conditions part of the selenium from proteins formed insoluble lead selenide which was filtered off with the lead sulfide, because all organic diselenides and several other organic selenium compounds give lead selenide when heated in alkaline plumbite (Painter, 1939). When hydrolysis is carried out in alkaline solutions without lead, the selenium cleaved from its organic linkage should first appear as alkali selenide. Part of the sulfur removed from cystine or proteins is oxidized and several inorganic sulfur compounds have been reported or suspected (Thor and Gortner, 1932-33; Clarke and Inouye, 1930). Diselenides give rise to alkali selenides and selenites in alkaline solution. If oxygen is present, inorganic selenides are decom-

TABLE II  
Selenium and Sulfur Distribution after Alkali Hydrolysis of Proteins

Hydroly- sate No.	Material hydrolyzed	Se p.p.m.	S per cent	Method of hydrolysis	PbS ppt.		Filtrate from PbS		Recovery	
					Se per cent	S per cent	Se per cent	S per cent	Se per cent	S per cent
9	Gluten 582	117	0.74	18 hrs. in 20% NaOH + PbO	33.3	52.8	65.0	46.4	98.3	99.2
10	" 607	90	0.64	18 " " 20% " + "	28.9	53.3	72.2	43.9	101.1	97.2
11	" 459	93	0.70	18 " " 20% " + "	33.3	57.2	59.1	41.7	92.4	98.9
12	Corn Protein 523	143	0.80	18 " " 20% " + "	23.8	33.1	82.5		106.3	
13	Barley Protein 585	94	0.73	18 " " 20% " + "	21.2	22.0	76.9		98.1	
14	Acid hydrolysate of Gluten 582*	95	0.56	18 " " 20% " + "		39.3	68.4	67.8		107.1
15	" " *	95	0.56	18 " " 5% " + "		41.6	54.8	60.7		102.3
16	" " †	20	0.32†	1 hr. " 20% " + "	40.0	50.0	55.0		95.0	
17	Gluten 607	90	0.64	18 hrs. " 20% " + " SnCl <sub>2</sub> §	37.8	69.2	55.6	33.2	93.4	102.4
18	" 582	117	0.74	56 hrs. in 6.5% Ca(OH) <sub>2</sub> + PbO	46.2	63.7	70.9	42.2	117.1	105.9
19	" 607	90	0.64	56 " " 6.5% " + "	32.2	57.8	66.7	45.3	98.9	103.1
20	Acid hydrolysate of Gluten 582*	95	0.56	18 " " 6.5% " + "	25.3	59.7	81.1	40.4	106.4	100.1
21	" " *	95	0.56	18 " " 5% NaOH			93.2	85.4		

\* Same hydrolysate as in Table I.

† Hydrolyzed with 18 N H<sub>2</sub>SO<sub>4</sub> (see Painter and Franke (1936)).

‡ Sulfur determined on an aliquot of the solution.

§ The method of Zahnd and Clarke was followed.

posed and metallic selenium is deposited when the solution is acidified.

In view of these properties it is rather surprising that more selenium is not filtered out from alkali hydrolysates in the absence of plumbite, especially when calcium and barium hydroxides are used, because with these alkalis there is much insoluble material upon which selenium may be adsorbed. If selenium is not separated at this point, one would expect that the metallic form would separate on acidification or by the action of reagents able to reduce inorganic selenite or selenate. Only a small fraction of metallic

TABLE III  
*Selenium Cleaved by Alkaline Hydrolysis*

Hydrolysate No.	Gluten No.	Method of hydrolysis	Total Se in insoluble residue, 1st hydrolysis	Se in PbS ppt., 2nd hydrolysis	Total Se recovered as inorganic Se
			per cent	per cent	per cent
22	582	18 hrs. in 20.0% NaOH	13.7*	3.0	16.7
23	582	56 " " 6.5% $\text{Ca}(\text{OH})_2$	15.4	9.4	24.8
24	607	56 " " 6.5% "	27.8	9.0	36.8
25	582	51 " " 14.0% $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$	6.0	13.7	19.7
26	607	51 " " 14.0% "	16.7	14.4	31.1
27	459	51 " " 14.0% "	14.0	18.3	32.3
28	583	51 " " 14.0% "	17.6	5.3	22.9

\* Obtained by acidifying and reducing with  $\text{SO}_2$  and hydroxylamine hydrochloride.

selenium has been obtained by reducing an acidified alkali hydrolysate of seleniferous proteins with sulfur dioxide and hydroxylamine hydrochloride.

The insoluble residue from calcium and barium hydroxide hydrolysates always contained some selenium, as is shown in Table III. After the insoluble material was filtered off, the hydrolysate was acidified with HCl and boiled for about 30 minutes. This treatment should oxidize alkali selenide to elemental selenium and remove the sulfide sulfur. The hydrolysates again were made alkaline with sodium hydroxide, lead oxide added, and the solutions boiled for 12 hours. A little lead-blackening sulfur was evident in every case, but there was much more in the



sodium hydroxide hydrolysate than in the barium hydrolysate, and only a trace in the calcium hydrolysate. The insoluble residue (lead sulfide + some organic material), which resulted from this second boiling in alkaline solution, contained selenium, as is shown in Table III.

Presumably the selenium in the insoluble residue from the first hydrolysis was cleaved to give alkali selenide and oxidized to elemental selenium. Selenide not determined in this fraction should have been recovered in the lead sulfide from the second hydrolysis in alkaline solution. It may seem surprising that in most cases the total inorganic selenium recovered in Table III does not equal that in the lead sulfide shown in Table II, but Painter (1939) found that all organic selenium compounds studied, except benzyl derivatives, decomposed to a greater extent when lead was present in the alkaline solution. Since it was difficult to filter out completely the metallic selenium in the acidified hydrolysates of organic selenium compounds (Painter, 1939), this may account for the variations in the results as well as some seemingly low yields of inorganic selenium. The greatest concentration of selenium in the protein hydrolysates was 0.00071 per cent. Colloidal selenium is estimated colorimetrically but it would be impossible to detect in the amber hydrolysates. If, after the lead sulfide was filtered out from the hydrolysate of a seleniferous protein, the rest of the lead was removed by addition of hydrogen sulfide, there was always a small percentage of selenium in the second lead sulfide.

It is assumed that the labile sulfur shown in Table II arises from cystine or cysteine. Blumenthal and Clarke (1935) give evidence that other sulfur compounds besides cystine and methionine occur in certain proteins; yet with the exception of djenkolic acid isolated by Van Veen and Hyman (1935), no other sulfur-containing amino acid has been isolated from plant proteins.

Although the lability of sulfur in the complex peptide linkages in proteins may be different from that of cystine or cysteine (Brand and Sandberg, 1926), the amount of lead sulfide formed in alkaline hydrolysis after reduction with stannous chloride > 6.5 per cent calcium hydroxide > 20 per cent sodium hydroxide; the same order as is obtained with cystine. The action of the different alkalis was, however, more pronounced with the acid hydrolysates than with the proteins.

By assuming that the amount of lead sulfide formed during the alkaline hydrolysis of proteins or acid hydrolysates approximates that from cystine by similar treatment (Zahnd and Clarke, 1933; Thor and Gortner, 1932-33; Clarke and Inouye, 1930), values for cystine are obtained which agree well with those reported by Jones, Gersdorff, and Moeller (1924-25), Looney (1926), and Baernstein (1932) for cereal proteins.

Most of the sulfur lost by hydrolysis of gluten by 33 and 50 per cent  $\text{H}_2\text{SO}_4$  is labile sulfur (compare Hydrolysates 9, 14, 15, and 16, Table II). There is much more sulfur in the humin when the stronger acid is used but a larger percentage of the total sulfur in the hydrolysate is labile sulfur. This may be explained by the work of Butz and du Vigneaud (1932-33) and Riegel and du Vigneaud (1935-36) who show that 50 per cent  $\text{H}_2\text{SO}_4$  converts methionine to homocystine or a thiolactone. Either of these compounds will give labile sulfur when heated in alkaline plumbite.

An intimate relationship between the mechanism of selenium and sulfur elimination from the protein molecule is suggested by the results. In most cases the percentage of selenium in the lead sulfide was increased by hydrolysis in solutions which removed a larger percentage of the sulfur from the protein. In every case, however, the percentage of total selenium in the lead sulfide precipitate was less than the percentage of total sulfur. Some selenium remains in the humin by acid hydrolysis, as is the case with sulfur, but when strong acids are used to hydrolyze the proteins the percentage of total selenium in the humin greatly exceeds that of sulfur (Hydrolysates 15 and 16, Table II, and selenium in humin). The presence of a diselenide is indicated but most diselenides give more lead selenide in alkaline plumbite (Painter, 1939). One selenium ether and one seleninic acid gave considerable lead selenide but seleninic acids also cleave in alkali to give selenite.

Recently Horn and Jones (1940) reported the isolation of a crystalline selenium compound from a plant source. Their product contains amino nitrogen and sulfur. The structure suggested by the empirical formula is of additional interest, because no analogous type of amino acid has been isolated. They report that the selenium in their compound is surprisingly stable, but not enough properties are given to show any relationship between their compound and the present work.

## EXPERIMENTAL

The wheat glutsens were obtained by salting-out crude wet gluten dispersed in 0.075 N acetic acid by the method previously described (Franke and Painter, 1936). The other proteins were extracted from finely ground grain with 0.2 per cent sodium hydroxide. After the protein solution had passed through a Sharples supercentrifuge, the protein was precipitated by neutralization. All analyses are reported on air-dried products.

The protein hydrolysate of the wheat gluten was obtained by sulfuric acid hydrolysis. The sulfuric acid was removed by barium hydroxide and the hydrolysate dried and kept in a desiccator.

TABLE IV  
*Determination of Sulfur in Lead Sulfide*

Source of PbS	Sulfur	
	Parr bomb method	Bromine-chlorate oxidation
	<i>per cent</i>	<i>per cent</i>
Protein hydrolysate.....	3.33	3.32
“ “ .....	1.90	1.80
Galena ore*.....	12.29	12.37

\* In the determination of sulfur on this sample by the method of Zahnd and Clarke it was necessary to add ammonium acetate to prevent the precipitation of lead sulfate.

In the hydrolysis of proteins, 1 gm. of protein was added to 20 ml. of sodium or calcium hydroxide solution or to 25 ml. of barium hydroxide solution. To insure an excess of lead, 1 gm. of lead oxide was allowed for every 10 gm. of protein. When stannous chloride was used, 1 gm. was added for every 25 gm. of protein, except when the procedure of Zahnd and Clarke (1933) was followed. Hydrolysis of wheat gluten with 7.6 per cent barium hydroxide indicated that approximately 50 hours boiling were necessary before the biuret test was negative. With 6.5 per cent calcium hydroxide, approximately 56 hours were necessary to hydrolyze the protein completely.

Usually 10 to 50 gm. of protein material were hydrolyzed be-

cause of a number of analyses to be made on each hydrolysate. After hydrolysis was complete, the materials were cooled, filtered by suction through a Buchner funnel, and made up to volume. The lead sulfide and other insoluble material were washed, dried, and weighed.

The use of the Parr bomb to determine sulfur in lead sulfide appeared more rapid than methods described. To check this method determinations were made on three samples with the bromine-chlorate method of Zahnd and Clarke (1933) and the Parr bomb method. The two samples of lead sulfide from protein hydrolysates have low sulfur values, because most of the weight was undissolved alkali. As high as 12 per cent sulfur has been obtained in lead sulfide formed from a protein in alkaline plumbite.

A sample of the hydrolysate equivalent to 0.5 gm. of protein material was pipetted into an evaporating dish, neutralized, and dried. Sulfur was then determined by the Parr bomb method.

All selenium determinations were made by the method of Robinson *et al.* (1934).

#### SUMMARY

Several seleniferous proteins and acid hydrolysates of seleniferous proteins have been hydrolyzed in alkaline solutions. The amount of selenium cleaved and recovered as inorganic selenium has been determined. When hydrolysis was carried out in the presence of alkaline plumbite, some selenium (probably as lead selenide) was in the lead sulfide. The selenium and sulfur distribution in the lead sulfide and hydrolysate was determined. If quantitative recoveries of cleaved selenium were obtained, the percentage of "labile selenium" was less than the percentage of "labile sulfur." It appears that the organic selenium compounds in proteins and in acid hydrolysates of proteins are more stable in basic solutions than are the sulfur compounds.

Selenium appears to be in essentially the same forms in the acid hydrolysates as in the original protein.

Acid hydrolysis of proteins results in a loss of labile sulfur but the loss of "labile selenium" exceeds the loss of "labile sulfur."

The destructive action of three alkaline solutions on cystine in proteins was in the order  $\text{Ca}(\text{OH})_2 > \text{Ba}(\text{OH})_2 > \text{NaOH}$ .

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## CHEMICAL AND METABOLIC STUDIES ON PHENYL- ALANINE

### III. THE AMINO ACID CONTENT OF TISSUE PROTEINS OF NORMAL AND PHENYLPYRUVIC OLIGOPHRENIC INDIVIDUALS. A NOTE ON THE ESTIMATION OF PHENYLALANINE\*

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The observation that the body fluids of phenylpyruvic individuals contain an abnormally large quantity of phenylalanine indicated that this disease was associated with an error in phenylalanine metabolism (1). Subsequently it was observed that fractional precipitation of the serum proteins with phosphate buffer of varying concentrations indicated a possible difference in the protein composition of normal and phenylpyruvic sera.<sup>1</sup> In the light of these observations, it was deemed advisable to investigate the amino acid composition, especially that of phenylalanine, of some tissue proteins prepared from phenylpyruvic oligophrenic and from normal persons. Therefore, brain, liver, kidney, and blood proteins were analyzed for nitrogen, sulfur, histidine, arginine, lysine, cystine, tyrosine, tryptophane, threonine, and phenylalanine.

#### EXPERIMENTAL

*Estimation of Phenylalanine*—In view of the importance of estimating as accurately as possible the proportion of phenylalanine present in the proteins, hydrolyses with various reagents were

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<sup>1</sup> Kondritzer, A. A., unpublished experiments.

carried out: 100 mg. portions of protein were heated under a reflux at 125° in an oil bath with 8 N sulfuric acid, 20 per cent hydrochloric acid-concentrated formic acid 1:1, concentrated hydriodic acid, 18 to 20 per cent hydrochloric acid, and 5 N sodium hydroxide. The time of heating was 2 hours. Phenylalanine was determined by the colorimetric method described recently (1, 2). The results of a few such experiments are given in Table I.

It will be seen that without exception the highest values were obtained after alkaline hydrolysis. However, the majority of phenylalanine estimations reported in Table II were made on hydrolysates prepared by heating with 8 N sulfuric acid overnight. Inspection of Table I shows that, although considerable variations in the percentages of phenylalanine were found by different methods of hydrolysis, a *comparative* analysis of normal and phenylpyruvic sera by any of the methods employed is capable of yielding significant results with the possible exception of hydrochloric acid-formic acid hydrolysis.

*Recovery of Added Phenylalanine*—Previous experiments (1, 2) indicate that all of the phenylalanine added to a protein hydrolysate is recovered by the *o*-dinitrobenzene method. However, this does not indicate how much of the amino acid is destroyed during hydrolysis. In an effort to obtain information of this nature, the following experiments were carried out. 200 mg. portions of gelatin or the same quantity of protein together with added phenylalanine was hydrolyzed with 8 N H<sub>2</sub>SO<sub>4</sub>, HCl-HCOOH, 20 per cent HCl, and 5 N NaOH as described above. The results of a few of these experiments, which are summarized in Table III, appear to confirm the superiority of alkaline hydrolysis.

*Amino Acid Composition of Some Proteins Prepared from Tissues of Normal and Phenylpyruvic Oligophrenic Individuals*—Blood was drawn from fasting normal and phenylpyruvic humans. The serum was removed by centrifugation and the proteins were isolated by heat coagulation or by precipitation with acetone. The lipids were extracted with hot alcohol, benzene, and ether and the white powdery proteins were dried at 110°. Proteins of the erythrocytes were extracted with organic solvents and dried in the same way. The serum proteins from twenty-nine normal and twenty-one oligophrenic individuals were used for nitrogen and phenylalanine estimations. The other amino acids reported in Table II were estimated on pooled specimens. The

values for normal serum are average figures from previous studies except in the cases of nitrogen and of phenylalanine.

TABLE I

*Phenylalanine Found on Hydrolysis of Proteins by Various Reagents*

	Hydrolyzing agent				
	H <sub>2</sub> SO <sub>4</sub>	HCl-HCOOH	HI	HCl	NaOH
	per cent	per cent	per cent	per cent	per cent
Cascin.....	3.9 (3)	4.9 (3)	4.3 (3)	5.0 (3)	5.8 (6)
Egg albumin.....	5.2 (3)	5.9 (3)	5.4 (3)	5.4 (3)	6.8 (6)
Lactalbumin.....	4.5 (4)	2.9 (3)	3.9 (3)	3.6 (3)	4.8 (6)
Gelatin.....	2.1 (12)	2.0 (12)	1.3 (12)	2.1 (12)	2.6 (12)
Normal serum....	5.0 (30)	6.1 (3)	4.9 (3)	5.0 (3)	7.0 (6)
Phenylpyruvic serum.....	5.3 (31)	5.3 (3)	4.9 (3)	5.4 (6)	7.1 (6)

The values in parentheses indicate the number of experiments.

TABLE II

*Amino Acid Composition of Some Proteins of Normal and Phenylpyruvic Oligophrenic Individuals*

Protein	Nitrogen	Histidine	Arginine	Lysine	Cystine	Tyrosine	Tryptophane	Threonine	Phenylalanine
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Normal serum*.....	14.9	2.4	4.5	7.0	3.6	4.1	0.4	5.8	5.0
Phenylpyruvic† oligophrenia, serum.....	14.9	2.1	4.4	6.2	3.1	4.3	0.4	5.6	5.3
Normal, red cells.....	16.1	6.1	2.6	6.2	1.0	6.5	1.2	5.7	8.5
Phenylpyruvic, red cells.....	15.5	5.6	2.5	5.8	1.0	5.8	1.0	5.2	7.2
Normal, brain.....	14.1	1.9	5.1	5.2	1.5	3.6	0.6	5.1	6.0
Phenylpyruvic, brain.....	14.3	1.9	5.4	5.3	1.5	3.4	0.6	5.4	5.7
Normal, liver.....	13.6	2.3	4.2	5.7	1.4	2.9	0.5	4.9	6.2
Phenylpyruvic, liver..	13.7	1.9	4.0	4.2	1.4	3.4	0.7	4.9	5.3
Normal, kidney.....	13.3	1.9	4.5	4.0	1.3	2.9	0.6	4.4	5.2
Phenylpyruvic, kidney	14.2	1.9	4.6	4.3	1.3	2.8	0.6	4.3	5.2

\* Sulfur, 1.46 per cent.

† Sulfur, 1.44 per cent.

The brain, liver, and kidney proteins were prepared from washed organs. These tissues were obtained at autopsy from one phenylpyruvic patient, a female 19 years of age. The normal tissue



proteins were similarly obtained from a female of approximately the same age and build as the patient.

All analyses were carried out two or more times. Nitrogen was determined by the macro-Kjeldahl method, sulfur by the Parr bomb procedure, and the amino acids by the methods described previously (3, 4).

The analytical results are summarized in Table II. The data do not show any significant differences in the amino acid composition of normal and phenylpyruvic blood and tissue proteins. Statistical treatment of the results obtained after  $H_2SO_4$  hydrolysis

TABLE III

*Recovery of Phenylalanine Added to 200 Mg. of Gelatin before Hydrolysis*

Hydrolyzing agent	Phenylalanine added	Phenylalanine recovered	Recovery
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
NaOH	2.5	4.9	96
"		2.5	
HCl	2.5	4.3	92
"		2.0	
HCOOH-HCl	2.5	4.2	92
"		1.9	
$H_2SO_4$	2.5	4.2	84
"		2.1	
HI	2.5	2.6	52
"		1.3	

All analyses were carried out six or more times.

of normal and oligophrenic serum proteins (by Dr. Joseph Zubin of the Psychology Department) indicates that these proteins do not differ significantly in phenylalanine content.

#### DISCUSSION

Westenbrink (5) in a recent review lecture says, "It will be clear that most proteins isolated from animal tissues are not characterized nearly sharply enough to provide an adequate material for investigations aiming at detecting physical or chemical differences between corresponding proteins from different species. We must confine ourselves to the whole protein complex of a certain organ or to some protein which happens to be highly

independent of the method of preparation." This statement is a concise summary of the ideas which we have pursued in this and previous investigations in comparative biochemistry (*cf.* (6) and earlier papers). The studies on keratins and serum proteins (orosins) are examples of Westenbrink's first class, while those on crystalline hemoglobins are examples of the second group.

Claims of differences in the composition of the same protein fractions among normal individuals of the same species or of purified proteins of normal and pathological individuals are still in a controversial state except in the case of the sulfur-containing amino acids. Roche (7) has claimed that the muscles of protein-starved rats show an increase in the content of the mono-amino acids and a decrease in tryptophane, tyrosine, and especially of lysine. The experimental data offered in proof of this statement are inadequate. Dirr (8) reported that the ingestion of arginine by normal individuals results in an increase in the arginine content of the serum proteins within a short time. These findings have not been confirmed (9). On the other hand the experiments of H. B. Lewis (10, 11) have shown clearly that the sulfur and cystine content of hair can be changed by dietary means. Alving and Mirsky (12) have also reported differences in the cystine contents of normal and pathological serum protein fractions, while Valer (13) has found differences in the sulfur content of crystalline hemoglobin prepared from different individuals of the same species.

In view of the reports in the literature cited above, it is of interest that the data presented in this paper show that the relative amounts of the nine or ten protein constituents determined are approximately the same in the blood, brain, liver, and kidney proteins of normal and oligophrenic individuals. In the case of the serum proteins, these findings are in accord with the electrophoretic (Tiselius) experiments of Dr. T. Shedlovsky and Dr. J. Scudder.<sup>2</sup>

#### SUMMARY

1. Several proteins were hydrolyzed with 8 N sulfuric acid, hydrochloric acid, hydrochloric acid-formic acid, hydriodic acid,

<sup>2</sup> Personal communication.

and 5 N sodium hydroxide. The results show that hydrolysis with sodium hydroxide gives the highest values for phenylalanine.

2. Proteins prepared from the sera, erythrocytes, brains, livers, and kidneys of normal and phenylpyruvic oligophrenic individuals were analyzed for their content of nitrogen, sulfur, histidine, arginine, lysine, cystine, tyrosine, tryptophane, threonine, and phenylalanine.

3. No significant differences were found in the amino acid composition of the proteins of the blood and tissues of normal and of phenylpyruvic oligophrenic persons.

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## THE EFFECT OF CASEIN AND A CASEIN DIGEST ON GROWTH AND SERUM PROTEIN REGENERATION

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But few observations have been reported in which the complete hydrolytic products of a protein were used for the regeneration of serum protein. Elman injected a casein digest intravenously in dogs after massive hemorrhage and observed slight increase in serum albumin after 6 and 24 hour periods (1). Over a period of years Whipple and coworkers have studied the effect of single amino acids on hemoglobin regeneration in their standard anemic dogs. A comprehensive survey of their findings has just appeared (2). Singly, each of sixteen amino acids was effective in increasing hemoglobin production; but certain chemicals, namely isovaleric acid,  $\beta$ -hydroxybutyric acid (or ester), glutaric acid, and even riboflavin (3), exerted the same effect. Their studies on dogs with lowered plasma protein (plasmapheresis) have resulted in the same type of finding. Thus, a mixture of cystine, glutamic acid, and glycine (4), or cystine alone (5), or other mixtures of amino acids containing cystine (6) resulted in large increases in serum protein when fed to dogs on a basal liver diet. Such findings raise in our minds the question of whether all such observed changes, resulting from incomplete mixtures of amino acids or particular chemical substances, are not solely the effect of a chemical stimulation, or mobilization of protein from body stores of protein, as distinguished from a new synthesis from the ingested protein materials. It was the object of this work to determine whether a digest of casein, consisting largely of amino acids, might promote as rapid growth and be as effective in the regeneration of serum protein as the unhydrolyzed protein. The experiments to be described indicate this was indeed the case.

The nutritive value of specific proteins has been determined in a variety of ways; *e. g.*, by the growth of experimental animals limited to the particular protein under investigation, by the amount required to maintain nitrogen equilibrium, and by the relative effectiveness in the regeneration of serum protein. Two methods are currently used to induce experimental hypoproteinemia, plasmapheresis and subsistence on a low protein diet. Plasmapheresis has been used largely by Whipple and Robscheit-Robbins (7) in their studies on both hemoglobin and serum protein regeneration. During observation the experimental dogs subsisted on a standard salmon bread diet. A modification (8) consists similarly in plasmapheresis (with differences in technique) but subsistence is on a protein-free diet.

The procedure of Weech and Goettsch (9) seems to us as somewhat more physiological. Dogs are maintained for a period of 3 weeks on a standard low protein diet. At the end of this period a drop in the level of albumin in the serum is noted. After a subsequent period of regeneration (with the protein and caloric intake constant) the increase in serum albumin is used as the index for assessing biological values.

Weech and Goettsch have compared beef serum protein, egg white, beef chuck, beef liver, casein, and gelatin for their ability to effect replenishment of a low serum protein (10). Considerable difference in effectiveness of the different proteins was noted; they are listed above in decreasing order of effectiveness. The authors show there is a difference of slight significance between serum protein and egg white, but that the differences between the former and the other proteins are highly significant. By recalculation from data given by Whipple and his associates, they show that findings with the two techniques are identical so far as the order of efficiency of the six proteins is concerned.

Rose (11) has summarized the cumulated experience with mixtures of amino acids, in their ability to support growth and maintain nitrogen equilibrium, in addition to his estimation of those acids essential and non-essential to the organism.

#### EXPERIMENTAL

To prepare the digest, casein was carefully washed to free it so far as possible from residual milk salts, lactose, and lactalbumin.

It was then digested at optimum pH and temperature with fresh pancreas extract until the amino acid content showed no further increase. Enzymic action was stopped, and the filtrate decolorized, concentrated, and spray-dried. After drying, the preparation contained 12.54 per cent total nitrogen and 7.41 per cent amino nitrogen (Van Slyke). In a sample boiled with concentrated acid, the amino nitrogen was 10.51 per cent. Complete hydrolysis by the enzyme had not been attained but all protein tests were negative (save for a slight biuret reaction), and it was impossible to sensitize guinea pigs to the dissolved material, or to produce shock with it in those sensitized to skim milk.<sup>1</sup> The preparation has been administered orally to infants as the sole source of nitrogen and positive nitrogen balances resulted (12). It has been injected intravenously in experimental dogs in large amounts without untoward effects,<sup>2</sup> although mild temperature reactions have sometimes been noted after its intravenous administration to infants (12).

*Growth of Rats*—Ten rats, 21 days of age, equally distributed as to sex and litter, were placed on each of eight diets containing 5, 10, 15, and 20 per cent of vitamin-free casein, or the casein hydrolysate, as the sole source of nitrogen save for the small amount contained in the brewers' yeast. The composition of the diets with 5 per cent casein or digest was as follows: dextrin 77 per cent, lard 9, salts (13) 4, cod liver oil 2, wheat germ oil 1, brewers' yeast powder 2, thiamine chloride 0.0006, riboflavin 0.0002. As the level of casein, or casein digest, was raised, the amount of dextrin was decreased proportionally.

The rats were kept on wire screens, offered the diet *ad libitum*, and weighed weekly for 8 weeks. There was a slight difference in the nitrogen contributed by the two supplements. The vitamin-free casein contained 15.40 per cent total nitrogen and the casein digest 12.54 per cent. In spite of this difference, the two materials were incorporated in the diets in equal and parallel amounts. The average weight curves are given in Fig. 1. The results are strictly comparable, because five male and five female litter mates were on each diet. At all levels of feeding the digest

<sup>1</sup> We wish to express our appreciation to Dr. P. S. Prickett and Norman J. Miller of this laboratory, for conducting these experiments.

<sup>2</sup> Unpublished observations in this laboratory.

gave larger weight gains than the corresponding level of casein; thus the differences in average weight after 8 weeks on the diets were at 5 per cent 1.1 gm., at 10 per cent 10.8 gm., at 15 per cent 30.8 gm., at 20 per cent 27.1 gm. The major portion of this superior gain was due to the more rapid growth of the males

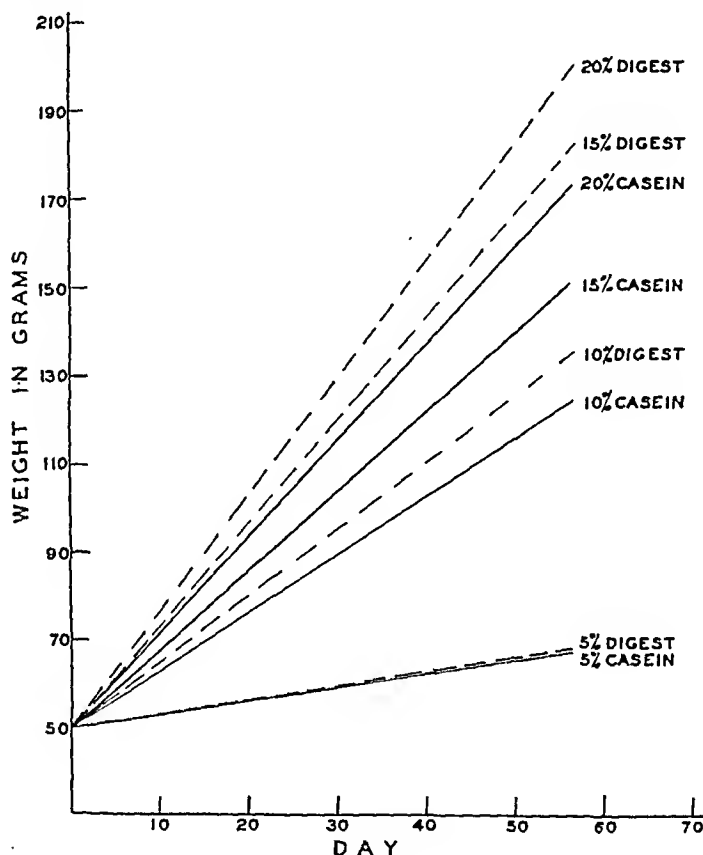


FIG. 1. Average growth curves of ten rats (equally distributed as to sex and litter) on each of eight diets containing the indicated percentage of casein or an enzymic digest of casein as the principal source of nitrogen.

receiving the digest. The males fed 20 per cent casein gained 6.3 per cent more weight than the females; on the digest they gained 23.2 per cent more; at 15 per cent intake comparable figures were 7.4 and 40.1 per cent; at 10 per cent intake, -2.8 and 10.2 per cent; at 5 per cent, -33.3 and -26.4 per cent respectively.<sup>3</sup>

<sup>3</sup> These differences merely indicate that the male uses a larger proportion of ingested nitrogen for growth than does the female. Similar observations have frequently been made (14).

The growth on 20 per cent casein and on 15 and 20 per cent of the digest was equal or superior to that observed on the stock diet and on a purified diet adequate for reproduction (15).

In a second series of rats weight records were supplemented with measurement of food intake and the determination of the nitrogen and water content of the rat carcasses after 8 weeks on the experimental diets. Ten 21 day-old stock rats, equally distributed as to litter and sex, on wire screens, were fed each of the two diets containing 15 per cent by weight of the nitrogenous component.<sup>4</sup> A record was made of the weight and food intake, and the latter corrected for any food spilled. After 8 weeks the rats were killed, decapitated, and the stomach and intestines removed. They were placed in individual, sealed, glass jars, cooked at 15 pounds of steam pressure for an hour, and then hashed in a meat grinder, with due precautions to get a uniform mixture. Total nitrogen and moisture were determined in triplicate.

The summarized findings are given in Table I. In this series, also, the rats fed the digest of casein gained more weight than their litter mates on unhydrolyzed casein. In terms of nitrogen, the casein-fed rats gained 9.75 gm. (s.d. 0.77) per gm. of ingested nitrogen,<sup>5</sup> while the rats fed the digest gained 11.88 gm. (s.d. 1.21) per gm. of ingested nitrogen. There was no essential difference in the composition of growth in so far as the nitrogen and water content of the rat carcasses was concerned. The average N content of the casein-fed rats was 3.40 per cent; of the digest-fed, 3.34 per cent. The solids content was 38.36 and 39.08 per cent, respectively.

The average differences in weight for comparable groups at the end of the experimental period have been subjected to statistical analysis. For the 20, 15, and 10 per cent levels the probability integrals<sup>6</sup> were 0.0598, 0.035,<sup>7</sup> and 0.246, respectively. Prevalent usage demands that the probability integral be 0.05 or less before

<sup>4</sup> The nitrogen content of the diet containing casein was 2.32 per cent; of the diet containing casein digest, 1.98 per cent.

<sup>5</sup> We make no effort to distinguish between the nitrogen that might have been used for maintenance and that used for growth.

<sup>6</sup> From tables of  $t$  given by Fisher (16).

<sup>7</sup> With both series of rats fed the 15 per cent level of casein or casein digest (*i.e.*, forty rats).



a significant difference can be assumed; to be highly significant the integral should be less than 0.01. Only one of the differences above (at the 15 per cent level) is less than 0.05. It is not possible to decide solely from growth during an 8 week period the relative nutritive value of two substances. However, the sup-

TABLE I  
*Food Intake, Weight Gain, and Carcass Composition of Rats Fed Either 15 Per Cent Casein or Casein Digest As Sole Source of Nitrogen for 8 Weeks*

Rat No. and sex	N source	Initial weight	Gain per day for 8 wks.	Total food intake	Gain per gm. N ingested	Per cent N in wet carcass	Per cent solids
		gm.	gm.	gm.	gm.		
81 ♀	Casein	52	2.00	586	8.22	3.40	40.54
82 ♀	"	44	2.36	573	9.92	3.42	39.91
83 ♀	"	47	1.79	459	9.38	3.38	40.33
84 ♀	"	49	1.93	467	9.95	3.15	36.70
85 ♀	"	52	1.93	510	9.12	3.34	40.26
86 ♂	"	47	2.14	490	10.55	3.54	39.68
87 ♂	"	53	1.86	443	10.10	3.41	36.91
88 ♂	"	47	2.46	611	9.73	3.50	35.96
89 ♂	"	51	1.93	488	9.53	3.40	36.60
90 ♂	"	57	3.02	661	11.01	3.46	36.66
91 ♀	Digest	50	2.16	527	11.58	3.27	39.11
92 ♀	"	43	1.62	444	10.35	3.21	40.11
93 ♀	"	48	2.04	475	12.11	3.15	39.07
94 ♀	"	48	2.34	524	12.63	3.14	37.85
95 ♀	"	54	1.89	563	9.50	3.34	36.56
96 ♂	"	46	2.61	647	11.39	3.89	40.47
97 ♂	"	49	1.55	361	12.15	3.58	36.30
98 ♂	"	45	3.20	613	12.75	3.03	39.79
99 ♂	"	50	2.37	500	13.43	3.27	39.03
100 ♂	"	57	3.27	716	12.91	3.47	42.49
Average for casein...		50	2.14	529	9.75	3.40	38.36
" " digest...		49	2.31	537	11.88	3.34	39.08

plementation of such figures with the finding that per gm. of nitrogen ingested the digest of casein gave considerably more growth than did casein itself strengthens the thought that the digest is nutritionally superior to the unhydrolyzed casein.

*Serum Protein Regeneration*—The basal nitrogen-low diet and the technique used throughout were those described by Weech

and Goettsch (9, 10). Three groups of dogs were made hypoproteinemic by feeding them the low proetin diet for 3 weeks; they were then fed supplements of either casein or casein digest for a regeneration period of 1 week. During the regeneration period each dog was fed daily 53.4 gm. per kilo of the basal diet. The control group received in addition that amount of casein (13.66 per cent N) required for a protein intake ( $N \times 6.25$ ) of 2.5 gm. per kilo of body weight. The protein content of the basal diet is given by Weech and Goettsch as 0.854 per cent,<sup>8</sup> so that 0.45 gm. of protein per kilo was furnished by the basal diet and 2.05 gm. per kilo were supplied by the casein. The weight of casein to supply this amount of protein was 2.40 gm. per kilo. The caloric intake was 80 calories per kilo of body weight.

The first experimental group received the casein digest (without regard to the nitrogen content) at a level calculated on the probably erroneous assumption that except for the moisture (3.7 per cent) and ash (4.5 per cent) the digest consisted solely of the hydrolytic products of pure protein. On this basis the supplement consisted of 2.23 gm. of hydrolyzed casein per kilo. In the second experimental group, feeding was based solely on equivalence in nitrogen intake. As the controls had received 0.327 gm. of nitrogen per kilo from the casein supplement, this group of dogs received a like amount of nitrogen from hydrolyzed casein, or 2.61 gm. of the supplement per kilo.

Total serum nitrogen and serum albumin were determined in duplicate on blood taken at the beginning, at the end of 3 weeks depletion, and after 1 week of regeneration. All nitrogen determinations were by the micro-Kjeldahl procedure. Non-protein nitrogen and amino nitrogen determinations were run on most of the samples but no significant change in any of these values was observed.

The essential data are presented in Table II. The assay values were calculated by adding a constant, 0.15, to the figures representing the gains in albumin in gm. per 100 cc. The magnitude of this constant was determined experimentally by Weech and Goettsch to compensate for the fall in albumin that would be

<sup>8</sup> The average protein content of twenty-five batches of the basal diet as made in this laboratory was  $0.638 \pm 0.011$  per cent. Actually, the basal diet contributed 0.34 gm. of protein per kilo.

TABLE II

*Assays of Casein and Casein Digest for Albumin Formation*

Dog No.	Serum albumin per 100 cc. plasma					Assay value
	Initial	Depletion	Regeneration	Loss	Gain	
Casein, 2.4 gm. = 0.327 gm. nitrogen $\simeq$ 2.5 gm. total protein per kilo body weight						
	gm.	gm.	gm.	gm.	gm.	
9	3.60	1.88	2.43	1.72	0.55	0.70
5	4.11	3.22	3.75	0.89	0.53	0.68
8	3.41	1.96	2.40	1.45	0.44	0.59
7	3.96	2.15	2.44	1.81	0.29	0.44
4	3.82	2.87	3.16	0.95	0.29	0.44
6	3.61	2.69	2.93	0.92	0.24	0.39
2	4.22	3.07	3.30	1.15	0.23	0.38
3	3.09	2.39	2.58	0.70	0.19	0.34
1	4.07	3.05	3.22	1.02	0.17	0.32
Potency value.....						0.476 $\pm$ 0.032
Casein digest, 2.23 gm. = 0.279 gm. nitrogen per kilo body weight						
	gm.	gm.	gm.	gm.	gm.	
1	3.03	2.44	2.95	0.59	0.51	0.66
5	3.72	3.11	3.53	0.61	0.42	0.57
2	3.38	2.68	3.02	0.70	0.34	0.49
4	3.40	2.51	2.78	0.89	0.27	0.42
10	3.46	2.61	2.82	0.85	0.21	0.36
3	3.42	2.43	2.61	0.99	0.18	0.33
9	3.64	2.72	2.84	0.92	0.12	0.27
6	3.80	2.92	3.04	0.88	0.12	0.27
7	3.52	2.44	2.51	1.08	0.07	0.22
8	3.82	2.06	2.12	1.76	0.06	0.21
Potency value.....						0.380 $\pm$ 0.032
Casein digest, 2.61 gm. = 0.327 gm. nitrogen per kilo body weight						
	gm.	gm.	gm.	gm.	gm.	
14	3.07	1.72	2.18	1.35	0.46	0.61
12	3.69	2.77	3.02	0.92	0.25	0.40
13	3.18	2.12	2.36	1.06	0.24	0.39
7	3.40	2.02	2.25	1.38	0.23	0.38
11	3.44	2.18	2.38	1.26	0.20	0.35
10	3.78	2.23	2.36	1.55	0.13	0.28
8	3.78	2.18	2.31	1.60	0.13	0.28
16	3.44	2.41	2.52	1.03	0.11	0.26
15	3.08	1.90	1.85	1.18	-0.05	0.10
Potency value.....						0.339 $\pm$ 0.031

expected during a 4th week of depletion in the absence of the casein supplement. The potency values are the averages of the assay values in each group.

The potency value for casein was slightly higher than for the casein digest: for casein,  $0.476 \pm 0.032$ ,<sup>9</sup> and for the two series with the casein digest,  $0.380 \pm 0.032$ , and  $0.339 \pm 0.031$ .

The differences are small and statistically unreliable, as can be seen from the estimates (probability integral) of the significance of the difference in potency value between casein and each series of the digests.

	Difference in potency values	Probability integral
Casein digest (lower level) and casein.....	0.096	0.175
" " (higher " ) " " .....	0.137	0.057

Since the probability integral in neither series is less than 0.05, it is necessary to conclude that there is no important difference in the regeneration of serum protein as effected by casein or a casein digest.

#### DISCUSSION

Published observations on protein digests have usually shown that such materials constitute adequate sources of nitrogen for growing animals, provided there is a deficiency of none of the essential amino acids and all toxic by-products of protein hydrolysis are absent. These conclusions can be confirmed from the work reported here. Technical perfection of a method to produce adequate quantities of a satisfactory digest was an essential part of the experiment, as the laboratory scale procedures usually employed are difficult to duplicate in successive trials, and usually yield materials of varying purity. By making a large amount of the digest it was possible to use the same material in different experiments without limitation because of inadequate supplies.

Observations on rats showed the digest was at least equal to the unhydrolyzed casein. The results suggest that it was superior. At four levels of intake, the average weight gain of the

<sup>9</sup> Probable error calculated as  $\sqrt{2d^2/(N-1)} \times 0.6745/\sqrt{N}$ .

digest-fed animals was greater than that of the casein-fed rats; and measurement of food intake at the 15 per cent intake level showed that per gm. of ingested nitrogen the digest gave larger gains in body weight than the unhydrolyzed casein. Analysis of the rat carcasses after 8 weeks on the experimental diets showed no important difference in total nitrogen or water content of the two groups. Thus the digest was fully available for metabolic purposes, and was as efficiently used as unhydrolyzed casein.

Serum protein regeneration is regarded as a fairly accurate index of the nutritional value of different proteins. We were unable to find any statistical difference in the effect of casein and casein digest in this regard. On the other hand, observations have been reported by Whipple and coworkers (2-7) that lactoflavin, isovaleric acid, and single amino acids result in serum protein or hemoglobin regeneration. The procedure we have employed, namely subsistence on a low protein diet and 1 week's regeneration, results in different potency values for different proteins (10) but observations on the effect of single amino acids or other chemicals have not been made. It is essential to know whether such materials, under the conditions of the experiment, will show protein regeneration. If they do, various modifications in the experimental procedure may have to be made. Such studies are now under way.

#### SUMMARY

1. The growth of 50 rats on four levels of casein has been compared with the growth of 50 litter mates fed identical levels of an enzymic digest of casein as the principal source of nitrogen. Food intake records showed that per gm. of nitrogen ingested casein gave 9.75 gm. gain in weight, whereas on the same basis the enzymic digest of casein gave 11.9 gm. gain in weight. The nitrogen and water content of the rat carcasses in the two groups was essentially the same. It was evident in all comparisons that the digest of casein was nutritionally equal or superior to casein itself.

2. The same two materials were used as supplements to effect regeneration of low serum protein induced in dogs by subsistence on a low protein diet. The average potency value obtained for the digest of casein was slightly lower than that for the casein

itself, but statistical analysis of the observed average differences indicates equality for the two substances in the regeneration of serum protein.

We wish to express our indebtedness to Dr. A. A. Weech for his assistance in the statistical interpretations.

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# THE APPEARANCE OF RADIOACTIVE IRON AS HEMOGLOBIN IN THE RED CELL

## THE SIGNIFICANCE OF "EASILY SPLIT" IRON

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It was pointed out recently that radioactive iron<sup>1</sup> could be demonstrated in the circulating red blood cells in a remarkably short time following feeding of the material to an anemic dog (6). Before we can be sure that it is present as *hemoglobin* iron and not in some precursor of the pigment, it must be demonstrated in the hemoglobin molecule. Routinely it would hardly be practicable to crystallize hemoglobin from all blood samples being studied, so several experiments were undertaken to determine the relation between the radioactive iron of hemoglobin and that of the red blood cell.

Many investigators now recognize a small fraction of the red cell total iron as non-hemoglobin iron. Barkan (2, 3) has extensively studied the so called "easily split" fractions of red cell iron. In order to convince ourselves that the iron appearing in the blood stream shortly after feeding is not in either of these fractions, these experiments were an essential step in our investigations of the iron isotope.

### *Methods*

Routine care of these anemic dogs has been described elsewhere in detail as well as methods for the determination of radioactivity

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<sup>1</sup> We are deeply indebted to Professor E. O. Lawrence and members of the Radiation Laboratory of the University of California for the radioactive iron used in these experiments, and in particular to Dr. M. D. Kamen who was directly responsible for preparing the isotope.



(1, 4-7). The radioactive iron used was prepared by the method described by Wilson and Kamen (13) at the Radiation Laboratory of the University of California.

Blood was drawn in isotonic sodium oxalate into 15 ml. centrifuge tubes, duplicate samples being taken in each instance. When there was activity in the plasma fraction, the cells were washed four times in saline and one of the samples of red cells wet-ashed with sulfuric and perchloric acids for subsequent determination of radioactivity. The duplicate sample, if being used for crystallization of the hemoglobin, was always washed four times with saline. Crystalline hemoglobin was then obtained by treating these cells according to the procedure of Welker and Williamson (12). The crystals were sucked as dry as possible on the filter and the drying completed in a desiccator containing concentrated sulfuric acid. The dry crystals were carefully removed from the filter paper, weighed, and transferred quantitatively to a digestion flask. From here on the procedure is identical with that used in preparing other material containing radioactive iron for counting.

The determinations of the radioactivity of the easily split iron fractions were carried out following treatment of washed red cells with an equal volume of acid (0.2 N hydrochloric, 0.2 N sulfuric, or 0.4 N sulfuric) in cellophane sacs according to the method as used by Moore (10).

Frequent hemoglobin determinations were carried out on the blood of these animals. From this and hematocrit readings, we are able to calculate the hemoglobin content of the red cells taken for crystallization. Knowing the weight of the hemoglobin crystals obtained and the amount of pigment from which they were derived, one can determine the yield of the procedure. Activity measurements of the crystalline material were then made after ashing and precipitation of the iron (4-7) and compared with the activity of the duplicate samples of red cells taken simultaneously, the latter being multiplied by the per cent yield of crystallization. In general the agreement was found to be fairly close (Table I). The values for a sample taken 5 hours after feeding show a wide discrepancy in one instance. This is explainable on a basis of the inaccuracy of measurement when the activity of samples is so near the "dark count."

The accuracy of measurement of the easily split fraction suffers somewhat because of the fact that only a small part of the total blood iron appears in the dialysate, and, if the total activity of the blood sample used is low, the accuracy of measurement of this fraction suffers accordingly. In such cases the accuracy probably is of the order obtained in chemical measurement of this small fraction, but not comparable with that usually possible in radioactive measurement.

## EXPERIMENTAL

In Table I are presented the essential data relating to the hemoglobin crystallization experiments. It is true that the crystals

TABLE I  
*Radioactive Iron in Crystalline Hemoglobin*

Dog No.	Time after feeding	Activity in red cells used	Yield of crystalline hemoglobin	Calculated activity in crystals	Observed activity in crystals
	<i>days</i>	<i>counts per min.</i>	<i>per cent</i>	<i>counts per min.</i>	<i>counts per min.</i>
38-112	10	83.5	38	31.7	33.3
37-202	1	13.2	29	3.8	3.9
	5	42	33	14	15
	7	36	25	8.9	11.0
37-202	0.2	0.8	31	0.3	0.7
	1	3.4	31	1.2	1.2
37-227	0.2	1.6	34	0.6	0.7
	1	8.3	26	2.1	2.1

obtained are not isoelectric hemoglobin, but the possibility of the radioactive iron being carried through the procedures and deposited quantitatively with the crystals as a salt or adsorption complex seems quite remote. This possibility will be considered again when we discuss the significance of the findings with the easily split fraction.

The values obtained for the easily split fraction of the red cells are in general agreement with those reported in the literature as regards magnitude. We must remember, however, that the *radioactive iron* in the blood in most instances is of *recent origin* in the red cell and these values are reported as the fraction of the *total red cell radioiron* found in this fraction and not the fraction

of the total cell iron. The time intervals following feeding at which these samples were collected were purposely varied and the samples were taken at chosen times when a variety of changes were taking place. The results may be seen in Table II.

TABLE II  
*Easily Split Fraction of Red Blood Cell Radioactive Iron*

Iron split off by	Dog No.	Iron dosage	Route of administration	Time after administration of radioactive iron	Time after last* phenylhydrazine injection	Per cent of total red blood cell radioactive iron as easily split
		mg.		days	days	
0.1 N hydrochloric acid	38-112	122	Intravenous	7		3
				13		7
					0	20
0.1 N sulfuric acid	38-146	17	Gavage	7	4	10
	38-182	160	Intraperitoneal	1		12
				5		21
				8		10
0.2 N sulfuric acid	37-202	4.9	Gavage	7		7
				1		15
				2		11
				5		17
	38-182	166	Intravenous	7		12
				3		5
				10		9
				12		10
	37-202	58	Gavage		-1	16
					3	23
					7	15
	37-202	58	Gavage	2		14
				5		6
				9		7

\* Acetylphenylhydrazine in series of five injections was administered subcutaneously to destroy the red blood cells. Time in days indicates the lapse of time between the last injection of the drug and the time of sampling.

#### DISCUSSION

It is apparent that the radioactive iron in the red cell is nearly quantitatively present in the hemoglobin crystals obtained from the cells. If it were present as non-hemoglobin iron but combined with stroma proteins, it would have been precipitated in whole or

for the most part by the aluminum cream used in the crystallization procedure. If it were in the form of a salt and carried down with the crystals, samples inspected for their easily split fraction taken at similar times would in all likelihood have shown nearly 100 per cent of their radioactive iron in the latter fraction, since the acid used in that procedure would almost certainly have enabled the iron to dialyze through the cellophane.

It has been suggested by Barkan and Schales (3) that the easily split fraction probably derives from pseudohemoglobin accompanying the formation of bile pigment, the iron and the verdohemochromogen (Lemberg (9)) being the split-products. Hawkins and Whipple have shown (8) that bile pigments derive from red cells which have reached the end of their life span, from traumatized cells, or from muscle hemoglobin, the amounts from each source not being exactly known. They estimate that about 0.8 per cent of the circulating red cells in the dog is broken down daily, giving rise to bile pigment. The remainder of the bile pigment formed in the dog through the other sources would be less than this according to their figures. The radioactive iron appearing in the easily split fraction in the experiments recorded in Table II was in every case present in new red cells and could therefore not be ascribed to breakdown due to aging of the cells. It would not be easy to conceive of the large amount of easily split radioactive iron demonstrated being the product of destruction of red cells by daily traumatization, since the amount would be far in excess of the daily breakdown as shown by the combined two possible sources of red cell trauma and muscle hemoglobin.

It is true that there is a suggestive increase in the easily split fraction following administration of acetylphenylhydrazine, but we must remember that a considerable amount of iron is being liberated during these periods, as shown by the liberation of large quantities of bile pigment, and the increase in the fraction of easily split radioactive iron would not seem to be great enough to account for this.

The easily split fraction of radioactive iron, which we know is present in very young red cells, is of the same order of magnitude as the easily split fraction of total red cell iron as found by Moore and his associates (10, 11).

These results do not substantiate the hypothesis of Barkan that the easily split fraction of red cell iron is associated with any

particular stage in the aging of the red cell. In fact, the evidence to date suggests that the easily split fraction may very well be an artifact.

#### SUMMARY

Radioactive iron demonstrated in the circulating red blood cells following its feeding is shown to be combined in the form of hemoglobin by radioactivity measurements of the crystallized pigment.

The radioactive element appears in the easily split fraction of the red cell radioiron in quantities ranging from 3 to 23 per cent of the red cell radioiron.

There is no obvious relationship between the amount of the radioactive iron in the easily split fraction and the time following administration of the isotope. Just as much was present in the newly circulating red cells 24 hours after iron feeding as weeks later.

There is some increase in the amount of radioactive iron in the easily split fraction following massive blood destruction due to acetylphenylhydrazine.

It is suggested that the easily split fraction of red blood cell iron is an artifact.

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## THE ISOLATION OF $\alpha$ -DIHYDROTHEELIN FROM HUMAN PREGNANCY URINE

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The quantitative determination of urinary estrogens is being applied widely as a research procedure in the study of normal and pathological conditions. Most investigators have limited their work to the estimation of total estrogenic potency, although it has been known for some time that the urine of pregnant women contains at least two estrogenic compounds, theelin and theelol.

Experiments upon rabbits by Pincus and Zahl (1937) indicated that theelin is converted to theelol by the action of progesterone upon the rabbit uterus. It appeared, therefore, that the separate determination of theelin and theelol in the urine of women might provide a gage of progestin as well as estrogen metabolism, and a study (Smith, Smith, and Pincus, 1938) in which this procedure was carried out during a normal menstrual cycle and a pregnancy gave evidence in support of the supposition. Further studies (Smith and Smith, 1938, 1940) have confirmed this observation and have demonstrated that the separate determination of urinary estrogens yields much more information than may be gained by the determination of total estrogenic potency alone.

In the interpretation of these data it becomes of importance to know whether or not any estrogens other than theelin and theelol are contributing significantly to the estrogenic potency of the separate fractions. Comparison of colorimetric with bioassay

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suggested the presence in the theelin fractions of late pregnancy urine of some estrogen other than theelin or theelol and much more estrogenically active in rats than either of them, possibly  $\alpha$ -dihydrotheelin (Smith, Smith, and Pincus, 1938). It was, moreover, discovered that semicarbazide treatment of the theelin fractions of urine from both pregnant and non-pregnant women failed to inactivate a large part of the estrogenic potency, 30 to 60 per cent in most specimens. Investigation of the quantitative accuracy of the method (Cohen and Marrian, 1934) used for separating theelin from theelol involved recovery experiments in which crystalline estrogens were added to non-pregnancy urines of low estrogenic content (Smith, Smith, and Schiller, 1939). It was found that no appreciable amounts of added theelol were taken into the theelin fractions, indicating that the potency of theelin fractions after semicarbazide treatment could be very little if at all attributable to theelol contamination. Further,  $\alpha$ -dihydrotheelin added to urine was completely recovered in the theelin fraction. These results again suggested the presence of  $\alpha$ -dihydrotheelin in human urine, accounting for the non-ketonic activity of theelin fractions. Only the ketonic activity of theelin fractions, therefore, was considered theelin, the rest being designated  $x$  estrogen.

If the so called  $x$  estrogen could be isolated and identified as  $\alpha$ -dihydrotheelin, the above interpretation of results would be considerably strengthened. In addition to the work in the Fearing Laboratory both Westerfeld and Huffman had worked on this problem because of interesting results that Westerfeld and Doisy obtained in 1937.

Certain reports on the isolation of  $\alpha$ -dihydrotheelin bear at least superficial evidence on the identity of  $x$  estrogen with  $\alpha$ -dihydrotheelin. In 1935, MacCorquodale, Thayer, and Doisy isolated this compound from sow ovaries. Of course, species differences do occur but in the absence of contradictory evidence it seems plausible to assume that the same compound is present in human ovaries. Owing to the difficulty of procuring sufficient human ovaries for examination, the investigation of this problem has not been completed but the estrogens of human placenta have been studied and  $\alpha$ -dihydrotheelin isolated and characterized (Huffman, Thayer, and Doisy, 1940). Furthermore, the isolation of this

compound from pregnant mare urine by Wintersteiner and co-workers (1935) and the report of the large proportion of non-ketonic estrogen in that source by van Stolk and de Lenchere (1937) indicated that the examination of human urine for dihydrotheelin might be attended with success.

Both laboratories were engaged in experiments designed to separate theelol and theelin from other estrogens of human urine at the time the Fearing group suggested to the St. Louis group that they would supply the partially purified extract for the isolation of  $\alpha$  estrogen.

At the outset of our attempt to isolate  $\alpha$ -dihydrotheelin from human pregnancy urine, it became apparent that rather specialized methods would have to be devised in order to separate this hormone from other non-ketonic estrogenic constituents (such as theelol) which might be present. It was at first tentatively proposed to approach the problem in this general fashion: (1) removal of ketones from the total phenolic extract of urine by means of carboxymethoxylamine; (2) protection of the phenolic hydroxyl in compounds of the remaining non-ketonic fraction by benzylation; (3) treatment of the benzoates of the non-ketones with lead tetraacetate under such conditions that monocarbinols would not react but under which the 1,2-glycol, theelol, would be oxidized to a dialdehyde; the latter product could then be separated from other carbinols by means of a suitable reagent; (4) saponification of the remaining monobenzoates, recovery of the free phenols, and ultimate isolation of the dihydrotheelin as the di- $\alpha$ -naphthoate.

It has been possible to realize experimentally all of the above steps with the exception of the quantitative monobenzylation of theelol. We have been unable to perform this step with micro quantities of theelol.

We next tried to adapt to our problem the method used by Whitman, Wintersteiner, and Schwenk (1937) for the separation of  $\alpha$ - and  $\beta$ -dihydrotheelin. According to Wintersteiner (1937, *a*), theelol and  $\beta$ -dihydrotheelin give no precipitate with digitonin in 80 per cent  $C_2H_5OH$ . Our adaptation of this procedure to the digitonin precipitation of micro quantities of dihydrotheelin was actually used at one stage of the isolation process reported in this paper.



Shortly after the experimentation mentioned above was completed, the discovery was made in the St. Louis laboratory by A. Mather that dihydrotheelin could be effectively separated from theelin by partitioning between benzene and 0.3 M  $\text{Na}_2\text{CO}_3$ . This discovery has, of course, greatly simplified our subsequent work.<sup>1</sup>

It is felt, however, that certain phases of our preliminary experimental work are of sufficient importance to warrant a brief discussion in this report.

### *Preliminary Experiments*

*Reaction of Theelin with Carboxymethoxylamine*—Although Westerfeld *et al.* (1938) had used Girard's reagent successfully in the separation of theelin from non-ketonic material, Wintersteiner (1937, b) reported that carboxymethoxylamine was superior to this reagent in the removal of ketones from mare pregnancy urine extracts.

The reaction of theelin with carboxymethoxylamine was first carried out according to the method of Anchel and Schoenheimer (1936); 15.2 mg. of theelin, 20 mg. of carboxymethoxylamine hydrochloride, and 54 mg. of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$  were dissolved in 4 cc. of 90 per cent  $\text{C}_2\text{H}_5\text{OH}$  and refluxed for 1 hour. The yield of carboxymethoxime was 78 per cent of the theoretical. Another similar experiment also gave a 78 per cent yield. Other reactions with smaller quantities of theelin were conducted, and bioassay upon the ether fractions (containing theelin which had failed to react) uniformly showed that this method did not give results which approached closely enough the quantitative to be suitable for our purposes. We, therefore, varied the procedure in several ways until an entirely satisfactory method was found.

A solution of 10.0 mg. of theelin, 24 mg. of carboxymethoxylamine hydrochloride, and 37 mg. of  $\text{KC}_2\text{H}_3\text{O}_2$  in 4 cc. of 75 per cent *n*-propyl alcohol (aldehyde-free) was refluxed for 3 hours. The solution was transferred to a separatory funnel with 100 cc. of 3

<sup>1</sup> In the recent isolation of  $\alpha$ -dihydrotheelin from human placenta (Huffman, Thayer, and Doisy, 1940) the benzene-carbonate distribution was employed successfully. It is likely that better results could have been obtained with the pregnancy urine had this method of separation been used. Actually at the time when we employed digitonin, Mather's research had not been completed.

per cent  $\text{NaHCO}_3$ ; the bicarbonate solution (which was water-clear) was extracted twice with 100 cc. portions of ether, and the latter then washed with bicarbonate and with water. Assay of the ether showed that less than 1 per cent of the theelin had failed to react. In Table I, additional data are given which show the degree of conversion of theelin into the bicarbonate-soluble derivative.

Theelin carboxymethoxime crystallizes from aqueous alcohol in beautiful white needles (m.p.  $188^\circ$ , uncorrected) which contain  $\frac{1}{2}$  molecule of  $\text{C}_2\text{H}_5\text{OH}$  of crystallization.

*Microcombustion Analysis*— $\text{C}_{20}\text{H}_{25}\text{O}_4\text{N} \cdot \frac{1}{2}\text{C}_2\text{H}_5\text{OH}$

Calculated, C 68.81, H 7.70; found, C 69.01, H 7.74

TABLE I

*Reaction of Theelin with Carboxymethoxylamine*

24 mg. of carboxymethoxylamine hydrochloride and 37 mg. of  $\text{KC}_2\text{H}_3\text{O}_2$  were refluxed in 4 cc. of 75 per cent *n*-propyl alcohol.

Theelin used	Time of refluxing	Theelin in ether phase by assay	Removal of theelin
$\gamma$	<i>hrs.</i>	$\gamma$	<i>per cent</i>
50.4	3	0.8	98.4*
230	3	1.0	99.5
298	5	0.7	99.8

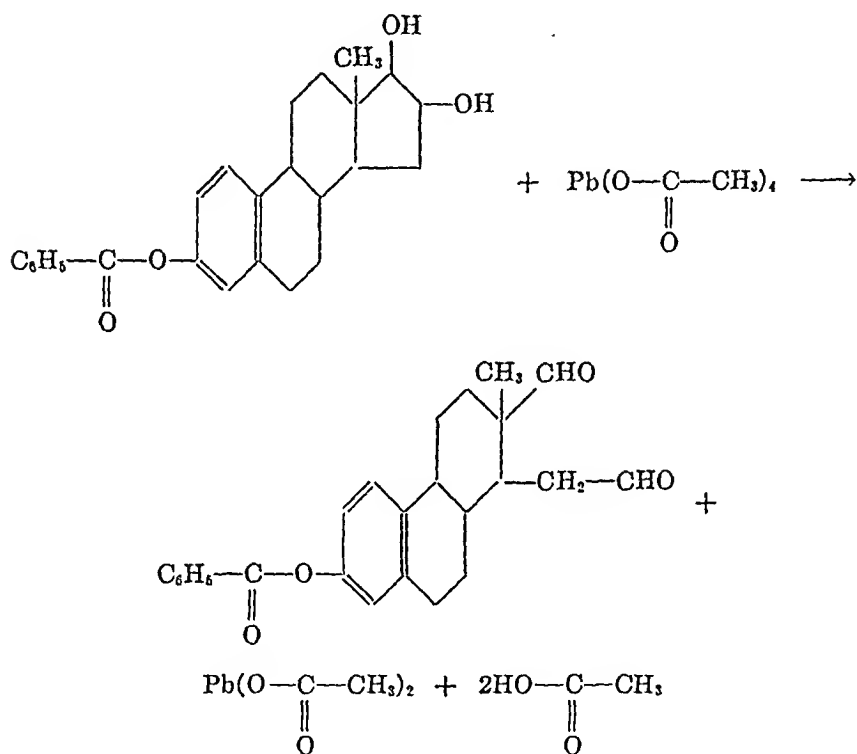
\* The failure to obtain better than 99 per cent removal in this case was probably due to the fact that the *n*-propyl alcohol had not been freed of aldehydes. Aldehyde-free solvent was used in the other runs.

*Hydrolysis of Theelin Carboxymethoxime*—A solution of 37.0 mg. of theelin carboxymethoxime (with  $\frac{1}{2}$  molecule of  $\text{C}_2\text{H}_5\text{OH}$  of crystallization) in 30 cc. of 1 *N*  $\text{HCl}$  + 30 cc. of 95 per cent  $\text{C}_2\text{H}_5\text{OH}$  was refluxed for 3 hours in a water bath with the temperature held at  $85$ – $90^\circ$ . Most of the alcohol was then distilled, water added to increase the volume to 100 cc., and the theelin extracted with 300 cc. of ether. The ether was washed once with 3 per cent  $\text{NaHCO}_3$  and twice with  $\text{H}_2\text{O}$ . Distillation of ether gave 24.4 mg. (theoretical 23.7 mg.). The crystalline material was treated with a small amount of norit and crystallized from aqueous alcohol; yield 21.8 mg. (92 per cent) of theelin.

Hydrolysis by this procedure of 8 micrograms of theelin carboxy-

methoxime showed a quantitative recovery of theelin, as determined by bioassay.

*Monobenzoylation of Theelol*—As stated before, we were unable quantitatively to obtain monobenzoyl theelol ( $C_3$  hydroxyl only), using micro quantities of theelol. However, theelol monobenzoate can be easily made by the ordinary Schotten-Baumann procedure.



It crystallizes from benzene in white platelets (m.p.  $225^\circ$ , uncorrected).

*Microcombustion Analysis*— $C_{25}H_{26}O_4$

Calculated, C 76.50, H 7.19; found, C 76.15, H 7.30

*Reaction of Theelol Monobenzoate with Lead Tetraacetate*—Lead tetraacetate reacts with theelol monobenzoate as indicated in the equation. Under the conditions of these experiments more than the theoretical amount of lead tetraacetate reacted. However, the aldehydic nature of the product was demonstrated by a positive Schiff's reaction and by the formation of a yellow hydrazone with phenylhydrazine. The reactive aldehyde group or groups

in the product make possible its complete removal from an ether solution by shaking with an aqueous solution of  $\text{NaHSO}_3\text{-Na}_2\text{SO}_3$ , an experiment which we have performed.

Theelol monobenzoate was dissolved in a 0.1 N solution of  $\text{Pb}(\text{Ac})_4$  in glacial acetic acid (redistilled from  $\text{CrO}_3$ ). The solution was allowed to remain in a glass-stoppered flask at  $20^\circ$  or  $30^\circ$  for several hours and then the excess  $\text{Pb}(\text{Ac})_4$  titrated in the manner described by Criegee (1931). Under these conditions  $\alpha$ -dihydrotheelin monobenzoate does not react. The results of typical experiments are shown in Table II.

*Precipitation of  $\alpha$ -Dihydrotheelin with Digitonin and Decomposition of Resulting Digitonide*—The method used follows the general outline of that given by Whitman, Wintersteiner, and Schwenk

TABLE II  
*Reaction of Theelol Monobenzoate with Lead Tetraacetate*

	Hormone used	Tempera- ture	Time	$\text{Pb}(\text{Ac})_4$ used
	m.eq.	$^\circ\text{C}$ .	hrs.	m.eq.
Theelol monobenzoate.....	0.260	30	14	0.462
" " .....	0.245	20	20	0.376
Dihydrotheelin monobenzoate ...	0.105	20	20	0.000
" " .....	0.097	25	18	0.000

(1937). A number of experiments were conducted to determine the modifications best suited to the most nearly complete precipitation of  $\alpha$ -dihydrotheelin in quantities of the order of a few mg. An example of the most satisfactory procedure is given as follows:

In a centrifuge tube 9.0 mg. of  $\alpha$ -dihydrotheelin and 80 mg. of digitonin (Merck) were dissolved with the aid of heat in 2.0 cc. of 80 per cent  $\text{C}_2\text{H}_5\text{OH}$ . The tube was stoppered tightly and left at room temperature for 2 days and then centrifuged at 3000 R.P.M. for 30 minutes, after which the alcohol was carefully drawn off with a capillary pipette. A small stirring rod was used to stir up the digitonide thoroughly with 3 to 4 cc. of absolute ether, and then centrifugation and removal of the supernatant liquid were carried out as before. This washing procedure was repeated. Finally the digitonide was dried by being allowed to remain at room temperature overnight.

The dried digitonide was dissolved in 0.5 cc. of dry pyridine and 8 to 10 cc. of absolute ether gradually added. The suspended material was packed by centrifugation and the supernatant fluid drawn off. In order to remove the sterol as completely as possible from the digitonin, the process was repeated twice. The combined solutions from the decomposition of the digitonide were added to 300 cc. of ether, and the ether washed once with 100 cc. of HCl (1:10) and five times with 50 cc. portions of water. All of the aqueous layers were combined, washed once with 100 cc. of ether, and the ether then washed three times with 50 cc. volumes of water. Combination of the two ether fractions and distillation to dryness gave a white product which after crystallization at a low temperature from a small volume of aqueous alcohol weighed 7.0 mg. and melted sharply at  $172^{\circ}$  (uncorrected). Assay of the 80 per cent  $C_2H_5OH$  and ether washings from the digitonide formation showed that between 1.0 and 1.5 mg. of dihydrotheelin had escaped precipitation, indicating therefore that with 9 mg. the completeness of recovery through the digitonide was about 85 per cent.

### *Experimental Work on Human Pregnancy Urine<sup>2</sup>*

*Extraction of Estrogens from Urine*—Urine from women during spontaneous labor and delivery<sup>3</sup> was chosen for the source, since the highest values for  $\alpha$  estrogen had been encountered in such specimens (Smith and Smith, 1940). Hydrolysis (by boiling under a reflux for 10 minutes with 15 per cent concentrated hydrochloric acid) and extraction with ether were performed within 12 hours of the time of collection. This precaution was taken in order to avoid the possibility of conversion of urinary estrogens upon standing.<sup>4</sup> Small batches of urine, 200 to 700 cc.,

<sup>2</sup> The hydrolysis of urine and the Cohen-Marrian separation were performed at the Fearing Research Laboratory by Smith, Smith, and Schiller. The remainder of the work reported in this paper was carried out by the St. Louis group.

<sup>3</sup> We are much indebted to Dr. S. B. Kirkwood, who arranged for the collection and refrigeration of specimens at the Boston Lying-In Hospital.

<sup>4</sup> Zine was not employed in any of the hydrolyses. It has been found that either addition of zine during acid hydrolysis or putrefaction of urine results in an increased yield of estrogens due in part to conversion of theelin into some compound of greater estrogenic potency (Smith, Smith, and Schiller, 1939).

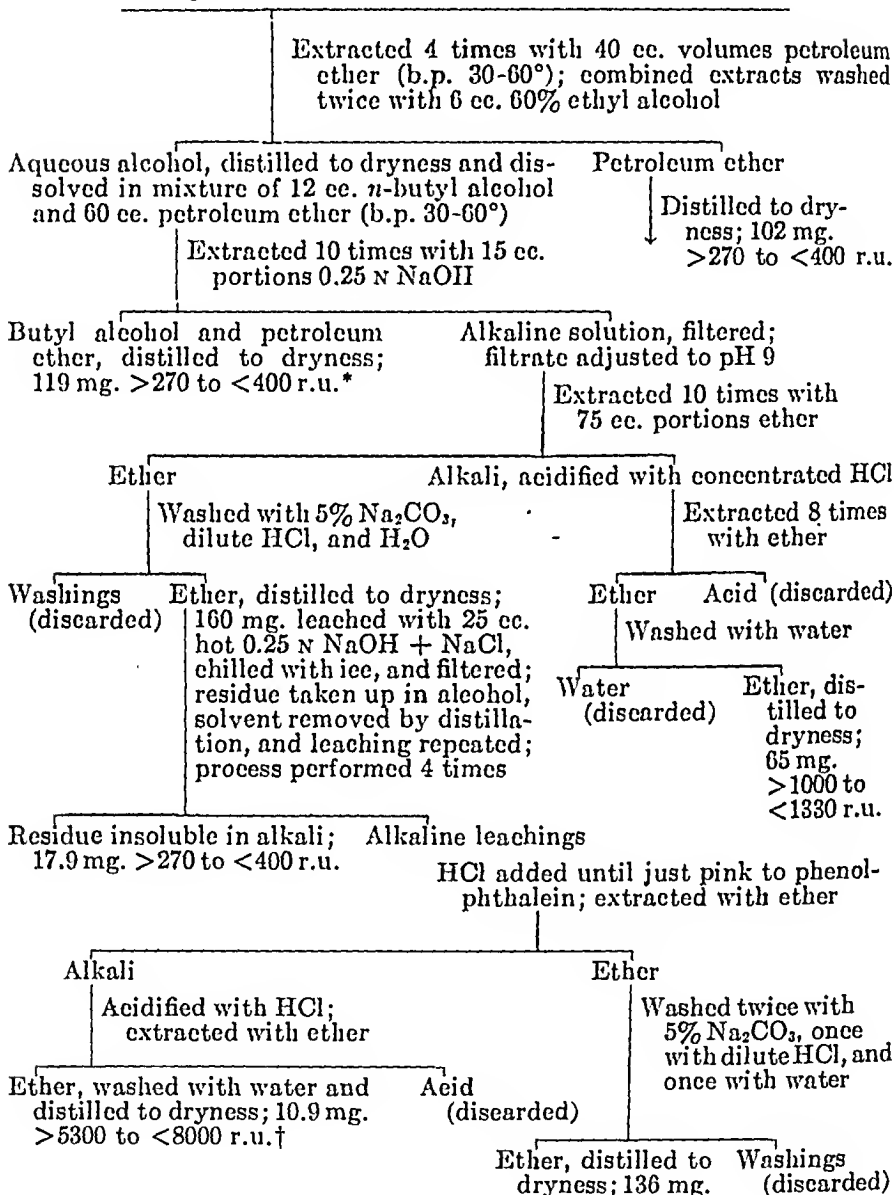
were handled at a time in order to get maximum recovery and more quantitative separation.

The weakly acidic phenols (theelin and  $\alpha$  estrogen fraction) were separated from the strongly acidic phenols (theelol fraction), according to the method of Cohen and Marrian (1934). They were then partially purified by reextraction from toluene, according to the Cohen and Marrian procedure, and stored in 95 per cent ethyl alcohol. Alcoholic solutions of the weakly acidic phenols from 38 liters of urine were combined and a small portion removed for bioassay before and after semicarbazide treatment. It was determined that the total extract contained 300,000 rat units of estrogenic substance, half of which represented non-ketonic ( $\alpha$  estrogen) material. If the non-ketonic activity were entirely attributable to  $\alpha$ -dihydrotheelin and the ketonic activity to theelin, it was calculated, according to the standardization values of the assay method used, that the extract contained 7.5 mg. of the former and 75 mg. of the latter.

*Preliminary Purification*—The extract of human pregnancy urine from the Fearing Research Laboratory was dissolved in alcohol and filtered through a Jena funnel. Evaporation of the filtrate gave 452 mg. of a dark orange-red material which by assay with mice showed estrogenic activity equivalent to 70 mg. of theelin. It was further purified as indicated in the flow sheet.

*Removal of Ketones*—The 136 mg. of material, which contained all but traces of the estrogenic activity, were dissolved in 20 cc. of 95 per cent  $C_2H_5OH$ , 160 mg. of carboxymethoxylamine hydrochloride and 420 mg. of  $NaC_2H_3O_2 \cdot 3H_2O$  (in solution in 2 to 3 cc. of  $H_2O$ ) added, and the whole refluxed for 3 hours. Most of the alcohol was then distilled off and the solution transferred to a separatory funnel with 15 cc. of 2 per cent  $NaHCO_3$  and 50 cc. of ether; the two phases were separated and the bicarbonate extracted twice more with ether. The combined ether solutions were washed once with 5 cc. of 2 per cent  $Na_2CO_3$ , three times with 5 cc. portions of water, and distilled; weight 100 mg. The 100 mg. of non-ketonic phenols were again treated with the ketone reagent but with refluxing for 7.5 hours. After this treatment the final non-ketonic fraction weighed 92 mg. (Fraction 01h). 27 mg. of crude theelin carboxymethoxime (Fraction

452 mg. solids in solution in 135 cc. acidified 70%  $C_2H_5OH$



\* The bioassays in this flow sheet were conducted at the Fearing Research Laboratory. 1 rat unit  $\approx$  0.5 to 0.667 microgram of theelin.

† After storage in a dry condition in a refrigerator for 13 months, assay in the St. Louis laboratory showed less than 1000 mouse units.

01f) were recovered from the aqueous bicarbonate and carbonate fractions.

*Isolation of Theelin*—Fraction 01f was hydrolyzed by the method given under "Hydrolysis of theelin carboxymethoxime;" yield 19.2 mg. of crude theelin. Treatments with norit and distribution between benzene and 0.3 M  $\text{Na}_2\text{CO}_3$  yielded 16.0 mg. in the benzene-soluble fraction. This material was naphthoylated with  $\alpha$ -naphthoyl chloride and attempts made to purify the theelin through crystallization of the  $\alpha$ -naphthoate, but a good product was not obtained. The crude  $\alpha$ -naphthoate and mother liquors from crystallizations were united and saponified with alcoholic KOH. The free phenol was then treated with semicarbazide and the semicarbazone recrystallized twice from aqueous ethanol ( $-5^\circ$ ). Hydrolysis of this apparently pure product was carried out by refluxing in 0.5 N HCl (50 per cent aqueous ethanol). After treatment with norit and one recrystallization of the phenol from aqueous alcohol, 3.79 mg. of white crystals, m.p.  $258-258.5^\circ$  (Anschütz), were obtained. A mixed melting point with authentic theelin (m.p.  $258.5-259^\circ$ ) was  $258.5-259^\circ$ .

*Microcombustion Analysis*— $\text{C}_{18}\text{H}_{22}\text{O}_2$

Calculated, C 79.95, H 8.21; found, C 79.98, H 8.44

*Isolation of  $\alpha$ -Dihydrotheelin*—Fraction 01h was assayed and found to possess activity equivalent to 50 mg. of theelin. It was dissolved in 95 per cent ethyl alcohol, concentrated to 1 cc., chilled, filtered, and the crystals washed once. This process was performed a total of three times; weight of crystalline fraction (01Hb) 25 mg. From these crystallizations and filtrations the filtrates (Fraction 01Ha) were combined, evaporated, dried, and weighed; weight 69 mg.

Fraction 01Ha was placed in a small Pyrex tube, 1.0 cc. of 80 per cent  $\text{C}_2\text{H}_5\text{OH}$  added, and the mixture warmed until solution was complete. Then 0.25 cc. of a solution of digitonin ( $\approx 10$  mg. of digitonin) in 80 per cent ethyl alcohol was added; in a short time a precipitate resembling a digitonide formed. Finally 0.75 cc. of 80 per cent  $\text{C}_2\text{H}_5\text{OH}$  and 70 mg. of solid digitonin were added, the mixture being warmed until all solids had dissolved. After the mixture had stood for 2 days, a very large precipitate



was observed, some of which appeared to be digitonide but most of which appeared to be digitonin. From this point on the procedure used is exactly as described under the heading "Precipitation of  $\alpha$ -dihydrotheelin with digitonin and decomposition of resulting digitonide." The yield from the decomposition of the digitonide was 5.2 mg. of a faintly yellow crystalline material (Fraction 01HaPD). Assays in mice indicated the presence of 2 to 3 mg. of dihydrotheelin and the assay in rats showed this product to be 3 times as active as pure theelin. A small aliquot of Fraction 01HaPD was distributed between benzene and 70 per cent  $C_2H_5OH$  and bioassay then conducted on each fraction. It was found that the activity was equally distributed in this partition. According to Westerfeld, Thayer, MacCorquodale, and Doisy (1938) the partition ratio of  $\alpha$ -dihydrotheelin between benzene and 70 per cent  $C_2H_5OH$  is 1:1.

The supernatant fluid and ether washings from the digitonide formation were evaporated to dryness and the reddish colored residue again treated with digitonin (20 mg. of digitonin in 2.0 cc. of 80 per cent  $C_2H_5OH$ ) but no precipitate occurred.

Fraction 01HaPD was acetylated and submitted to fractional distillation in a molecular still, but no substantial concentration of the active material (as determined by bioassay) could be realized. The acetylated material was then saponified, and the phenol recovered and partitioned between 0.3 M  $Na_2CO_3$  and benzene. From the benzene, 2.11 mg. of white crystals were obtained. These crystals were naphthoylated with  $\alpha$ -naphthoyl chloride (MacCorquodale, Thayer, and Doisy, 1936) to give 3.31 mg. ( $\approx$  1.50 mg. of  $\alpha$ -dihydrotheelin) of a compound melting at  $196.5-197^\circ$  (corrected). A mixed melting point taken with an authentic sample of  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate (m.p.  $198-198.5^\circ$ , corrected) was found to be  $197-198^\circ$  (corrected). A mixed melting point taken with an authentic sample of theelin  $\alpha$ -naphthoate ( $210^\circ$ , uncorrected) was  $180-185^\circ$  (uncorrected).

*Microcombustion Analysis*— $C_{40}H_{36}O_4$

Calculated, C 82.72, H 6.25; found, C 82.37, H 6.13

*Isolation of Theelin*—Fraction 01Hb was united with Fraction 01HaNPD (the portion of No. 01Ha remaining after precipitation with digitonin) and dissolved in 74 cc. of 95 per cent  $C_2H_5OH$ ;

26 cc. of water were added, and the aqueous alcohol was then extracted three times with 25 cc. portions of petroleum ether (b.p. 30–60°). Distillation of the aqueous alcohol gave a nice crystalline product with a brown contaminant. This material was dissolved in 500 cc. of 0.3 M  $\text{Na}_2\text{CO}_3$  and then extracted once with 500 cc. of benzene.<sup>5</sup> The carbonate fraction was extracted three times with 400 cc. of ether, after which the combined ethers were washed three times with water and distilled to dryness; yield 35 mg. which by assay indicated the presence of approximately 18 mg. of theelol.

The 35 mg. of crystalline material were acetylated with acetic anhydride, and the acetylated product distilled in a molecular still at 110–130° for 5 hours, and then at 180–200° for another period of 5 hours (pressure 0.0001 mm.). Saponification of the distillate gave crystals with very little color. These were crystallized twice from 1 to 2 cc. volumes of absolute acetone (–5°), treated once with norit, and finally recrystallized from aqueous methanol; yield 9.02 mg., melting at 279° (Anschütz). A mixed melting point with authentic theelol (m.p. 282–283°) was 280–282°.

*Microcombustion Analysis*— $\text{C}_{18}\text{H}_{24}\text{O}_2$

Calculated, C 74.95, H 8.39; found, C 74.92, H 8.32

#### SUMMARY

1. A method has been devised by which the ketonic estrogen, theelin, reacts quantitatively with the ketone reagent, carboxymethoxylamine. The resulting methoxime (which has been characterized) is soluble in aqueous bicarbonate and can thus be quantitatively separated from an ether solution of non-ketonic estrogens.

2. Theelol monobenzoate has been prepared and characterized; it reacts with lead tetraacetate in the usual fashion of 1,2-glycols.

3. The method of Wintersteiner *et al.* for the precipitation of  $\alpha$ -dihydrotheelin with digitonin and the decomposition of the resulting digitonide has been adapted for work with micro quantities.

<sup>5</sup> The 500 cc. of benzene contained between 1 and 2 mg. of dihydrotheelin by bioassay.

4.  $\alpha$ -Dihydrotheelin has been isolated from human pregnancy urine collected during spontaneous labor and delivery; the  $\alpha$ -dihydrotheelin was isolated as the di- $\alpha$ -naphthoate and characterized as such by the melting point, by the mixed melting point with authentic  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate, and by microcombustion analysis.

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## THE RELATION OF THIAMINE TO CITRIC ACID METABOLISM\*

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The discovery of the identity of thiamine pyrophosphate and cocarboxylase and the recognition of the ester as the predominant form of thiamine in yeast (1, 2) and animal tissues (3, 4) has been followed by several papers which have clearly demonstrated that cocarboxylase is essential for the oxidative metabolism of pyruvic acid (5-7). However, it has become clear that pyruvic acid is one of the most reactive intermediates in carbohydrate metabolism and that the end-products of its oxidation may vary with different organisms or tissues and with the internal conditions of the cell (6). Thus, it has been demonstrated *in vitro* that the oxidation of pyruvic acid under various conditions may yield acetic acid (6, 8, 9), acetic and formic acids (6), lactic and acetic acids (9, 10), acetoacetic and  $\beta$ -hydroxybutyric acids (11), succinic acid (9, 12), and citric acid (13, 14). Lipmann (5) and Barron and Lyman (6) have demonstrated with bacteria that cocarboxylase is essential for the aerobic oxidation of pyruvic acid to acetic acid, and for the anaerobic dismutation to acetic and lactic acids. Banga, Ochoa, and Peters (7) have demonstrated that thiamine pyrophosphate is essential for the oxidation of pyruvate by brain. While the over-all effect of cocarboxylase upon the oxidation of pyruvic acid is beyond question, its function in the specific oxidative transformations is not yet clear. In the absence of thiamine, it appeared likely that some of these reactions might be significantly affected.

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As a result of experiments *in vitro*, Krebs and Johnson (15) have proposed that the citric acid cycle is of great quantitative importance in the metabolism of carbohydrate by animal tissues. Krebs (16) has also reported that rats which are deficient in thiamine excrete 3.0 gm. of citric acid per kilo of body weight per day. In view of the great loss of weight observed during the production of thiamine deficiency and the small quantities of urine excreted during this condition, the data of Krebs suggest a figure of from 3 to 5 per cent citric acid in the urine of thiamine-deficient rats. Qualitative confirmation of these data has been obtained by Krusius and Simola (17) who report that the daily urinary excretion of citric acid by rats on a vitamin B complex deficiency rises from 0.34 to 4.49 mg. after 10 days upon the deficient ration.

The experiments of Krusius and Simola (17) are difficult to evaluate, since the ration was not specifically deficient in thiamine. The paper of Krebs (16) contains no information of the nature of the ration, particularly with regard to citric acid content or to adequacy in the other factors required by the rat.

The possible importance of the citric acid cycle in the aerobic oxidation of carbohydrate, the magnitude of the citric acid excretion described by Krebs, and the implications of his work with respect to the rôle of thiamine have prompted the present investigation. Our data fail to confirm those of Krebs, since we find a small decrease in citric acid excretion during acute thiamine deficiency, and a very large rise in the excretion of this compound when thiamine is administered to deficient animals. A preliminary report of this work has been previously presented (18).

#### EXPERIMENTAL

Albino rats, 3 weeks of age and weighing 40 to 50 gm., were placed on a ration deficient in thiamine and low in citric acid, but containing adequate amounts of all the other growth factors. The composition of the ration is as follows: sucrose 61, casein (vitamin-free, Labco) 18, autoclaved peanuts (5 hours at 15 pounds) 10, autoclaved yeast (5 hours at 15 pounds) 4, sulfite-treated liver extract 3, Salts 3 (19) 4, haliver oil 2 drops weekly.

The liver extract was prepared by adding 5.8 per cent  $\text{Na}_2\text{SO}_3$  to commercial 95 per cent alcohol-soluble liver extract (The Wil-

son Laboratories) at pH 4.8 for 12 hours. This procedure destroys thiamine completely (20). Acute polyneuritis is produced on this ration in 4 to 6 weeks. Control rats fed this ration with added thiamine (200 micrograms per 100 gm. of ration) showed normal growth, indicating that the ration is complete in all factors required by the rat. The citric acid content of this ration is 1.3 mg. per gm.

The rats were housed in wire-bottomed metabolism cages each suspended over a large glass funnel. The urine, separated from the feces by fine window screening, was collected under toluene, measured, and stored in the refrigerator until analyses were made. Weight, food consumption, and water consumption records were taken daily.

Control rats were fasted in the metabolism cages for 24 hours once a week in order to determine the effect of food intake upon citric acid excretion.

Synthetic thiamine, obtained from Merck and Company, Inc., was administered intraperitoneally or was dried on the ration. Sodium succinate solution was injected intraperitoneally at a level of 10 mm per kilo of weight.

Citric acid was determined by the method of Pucher, Sherman, and Vickery (21) adapted for the Evelyn photoelectric colorimeter.

### Results

*Citric Acid Excretion in Thiamine Deficiency*—The citric acid excretion of control, fasted, and polyneuritic rats is given in Table I. The values for the thiamine-deficient rats represent the period during which convulsions were observed. It may be seen that the average excretion of citric acid during acute polyneuritis is significantly lower than that of control rats receiving the same ration plus adequate thiamine. A typical experiment is shown in Fig. 1. As the animal is maintained upon the deficient ration, citric acid excretion falls and then shows a transitory rise before acute symptoms are evident. This rise is followed by a consistent drop in the citric acid excretion until convulsive symptoms appear. Since random samples were taken for analysis during the period of vitamin depletion, and daily samples were taken only for the week immediately preceding the appearance of acute deficiency symptoms, the transitory rise may have no significance. On the

other hand, the definite daily drop observed in all animals following this period and the low values observed during the acute deficiency appear to justify the conclusion that citric acid excretion is below normal during acute thiamine deficiency. These results are contrary to those observed by Krebs (16). The early transitory rise observed during the production of the deficiency may be similar to that reported by Krusius and Simola (17).

TABLE I

*Citric Acid Excretion by Control, Fasted, and Thiamine-Deficient Animals*

The results are expressed in mg. of citric acid per kilo per day.

Control			Fasted 24 hrs.		Thiamine-deficient	
Rat No.	Duration of period	Average daily excretion	Rat No.	Average* daily excretion	Rat No.	Average† daily excretion
	days					
1	20	15.7	9	30.69	1	10.63
2	25	21.2	10	78.18	1a	23.67
3	27	25.0	11	41.15	2	23.16
4	11	53.0	12	53.80	3	20.74
					3a	0.33
					4	9.06
					7	8.09
					8	5.82
					6	7.73
					20	12.3
					30	20.3
					16	16.2
Average .....		28.7		50.96		13.17

\* These figures are the average of three 24 hour fasts once a week.

† These values represent the excretion of citric acid while convulsions were evident. In every case they are the average of at least 2 days.

Since our experimental ration contains small amounts of citric acid, the urinary citrate may not be entirely endogenous. However, the normal animal is able to metabolize large amounts of ingested citric acid (22-24). At the beginning of the experiment, the excreted citric acid represents but a small fraction of the total intake. As the deficiency progresses, it will be apparent that the citrate intake and output tend to approach each other. The transitory rise in citrate excretion may indicate decreased ability

to metabolize this compound. The fall in the urinary excretion of citric acid is accompanied by a considerable decrease in food consumption, which cannot be entirely due to inanition since fasted control animals showed increased citrate excretion.

It has been demonstrated (13, 14) that citric acid is produced as a result of the condensation of oxalacetic acid and pyruvic acid followed by oxidative decarboxylation of the resulting 7-carbon intermediate. If thiamine were not essential for this reaction, it would be expected that the increased pyruvate characteristic of the vitamin deficiency (25-27) would lead to increased

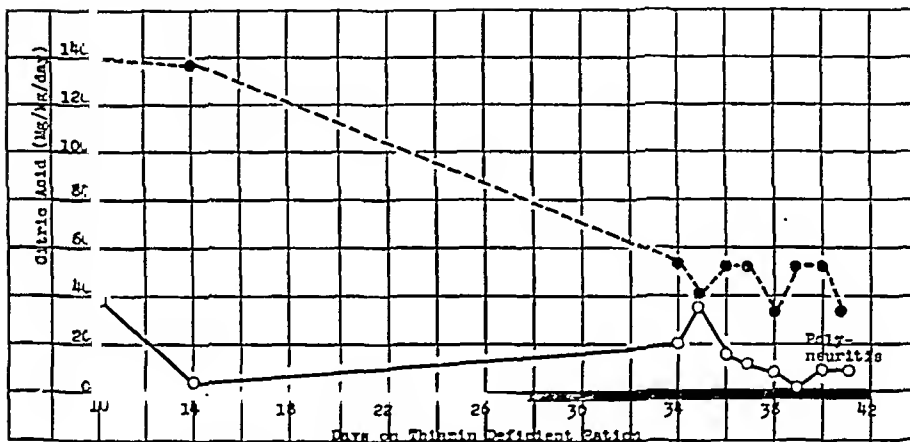


FIG. 1. The effect of thiamine deficiency upon urinary citric acid excretion. The dash line represents citric acid intake calculated from food consumption; the solid line, citric acid excretion.

citric acid formation. The fact that the citric acid excretion is below normal during the acute deficiency appears to indicate that thiamine is essential for this transformation. Similar conclusions have recently been suggested by Hallman and Simola (13).

*Citric Acid Excretion Following Succinate Injection during Thiamine Deficiency*—Smith and Orten (28) have shown that succinic acid injection into normal animals increased the excretion of citric acid greatly. The increase is of short duration, since the citrate excretion returns to normal within 6 to 8 hours. This has been confirmed by Krebs, Salvin, and Johnson (29) and has been incorporated by them as evidence for the citric



acid cycle. According to their view, injected succinate is rapidly converted to fumaric, malic, and finally oxalacetic acid. The latter compound condenses with pyruvic acid as previously described to yield citric acid ultimately. Our previous results appeared to indicate that thiamine was essential for the synthesis of citric acid. In an effort to test this further, animals deficient in thiamine, but as yet exhibiting no convulsive symptoms, were injected with 10 mm of sodium succinate per kilo of weight on alternate days until acute polyneuritis was evident. Fig. 2 shows that the ability of a typical animal to convert succinate to

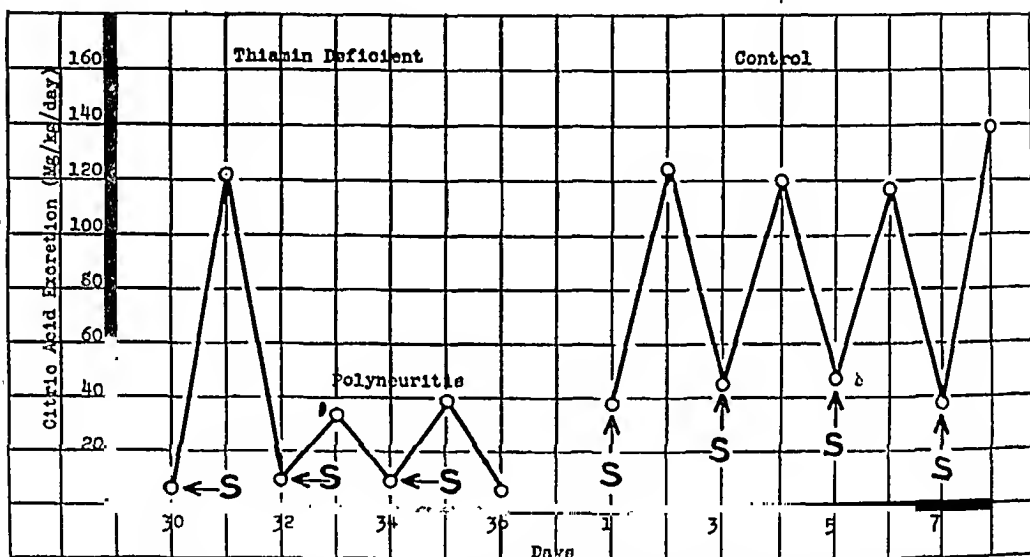


FIG. 2. The effect of succinate injection upon citric acid excretion by thiamine-deficient and control rats. S, 10 mm of sodium succinate per kilo injected intraperitoneally.

citrate diminishes with increasing severity of polyneuritis. Control animals treated exactly the same way do not show this effect. Table II summarizes the results obtained with normal and thiamine-deficient animals when injected with succinate. The values for the deficient rats are for the period during which acute deficiency symptoms were evident. It may be seen that the increment of citric acid excretion due to succinate injection is considerably lower in thiamine deficiency than in control animals.

*Citric Acid Excretion Following Thiamine Therapy*—In order to test further the relationship of thiamine to citric acid metab-

olism, curative experiments were employed. Deficient rats were cured by a single injection of 100 micrograms of thiamine.

Following such therapy there is a progressive rise in citrate excretion with maximum values being reached in 4 to 6 days. At this time the citrate excretion is approximately 10 times that of normal. The citrate excretion then drops more slowly and normal values are restored within 12 days following therapy. Similar results have been obtained in experiments in which polyneuritis has been cured by supplementation of the ration with 200 micrograms per cent of thiamine. A typical experiment showing the rise in citrate excretion following both forms of therapy is shown in Fig. 3. The progressive rise in citrate excretion may

TABLE II

*Average Increase in Citric Acid Excretion Due to Single Injection of Succinate*

1.0 ml. of M Na succinate per 100 gm.

The results are expressed in mg. of citric acid per kilo per day.

Control		Thiamine-deficient	
Rat No.		Rat No.	
3	75 (7)*	16	34 (2)
22	90 (7)	17	9 (3)
24	62 (7)	2	18 (3)
25	56 (5)	15	20 (2)
Average.....71			20

\* Figures in parentheses represent the number of injections of sodium succinate.

be noted in both cases. However, the rise and the return to normal are more rapid following the injection of a large single dose of thiamine. In addition, higher values of citrate excretion are obtained with the injection technique.

Table III shows the maximum daily citrate excretion of a number of polyneuritic rats following vitamin therapy. In every case, citric acid intake calculated from the food consumption demonstrates that at the peak of the excretion there is a greater urinary output than can be accounted for by the ingested citric acid. The lack of correlation between the intake and excretion of citric acid is further demonstrated by the fact that the drop to normal occurs despite the maintenance of food consumption.

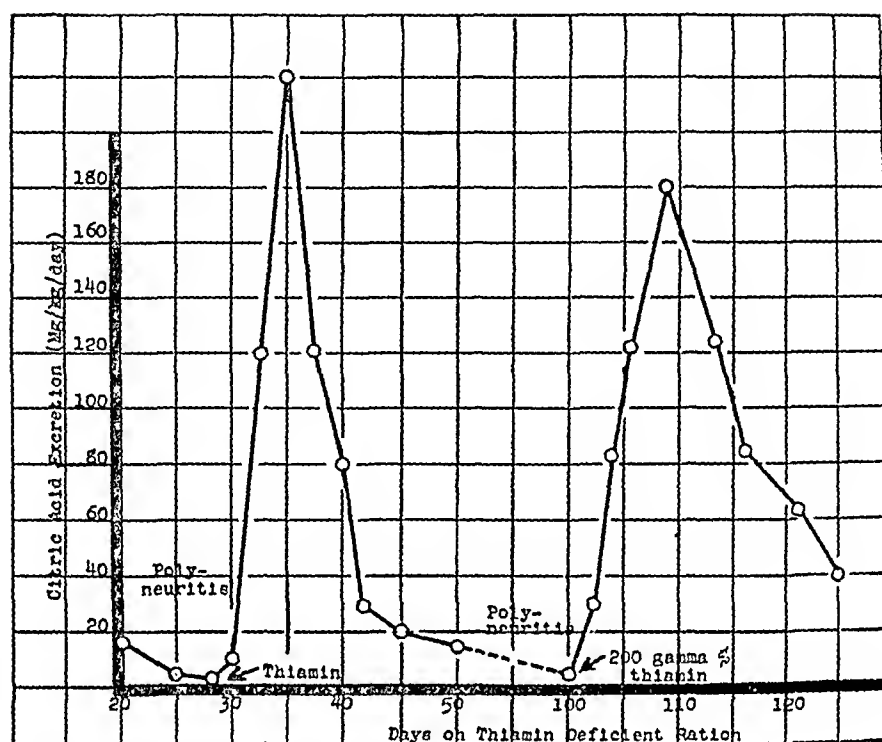


FIG. 3. The effect of thiamine therapy upon citric acid excretion of thiamine-deficient rats. At 28 days 100 micrograms of thiamine hydrochloride were injected intraperitoneally; at 100 days the basal ration was supplemented with 200 micrograms per cent of thiamine.

TABLE III

*Effect of Thiamine Therapy on Citric Acid Excretion of Thiamine-Deficient Rats*

The results are expressed in mg. of citric acid per kilo per day.

Rat No.	Excretion before therapy	Maximum excretion after therapy	Intake during maximum excretion	Days after therapy
1	3.99	287	165	4
2	14.5	299	136	5
3	5.7	227	125	4
	4.4	348	98	5
	4.0	213	167	9*
4	5.2	299	140	4
8	7.9	272	161	6

\* This animal was cured by supplementation of the ration with 200 micrograms per cent of thiamine. All other animals were injected with 100 micrograms of thiamine.

## DISCUSSION

The work of Martius and Knoop (30-32), Simola (33, 34, 13), and Krebs (15, 35) appears to have demonstrated that citric acid plays an important rôle in the metabolism of carbohydrate. The cycle proposed by Krebs and Johnson (15) for most tissues has been questioned by Breusch (36) who has been able to demonstrate citric acid synthesis *in vitro* only with kidney tissue (36). However, Hallman and Simola (13) have demonstrated that citric acid can be synthesized from malic and pyruvic acids by heart muscle *in vitro* and it has been demonstrated that other tissues show appreciable citric acid synthesis in the presence of oxalacetic and pyruvic acids (34). More recently, Krebs (14) has reaffirmed the existence of the citric acid cycle in pigeon breast muscle, cardiac muscle, and kidney. In these organs, this cycle appears to be an important mechanism for the metabolism of pyruvic acid.

Two oxidative decarboxylations are required in the Krebs cycle. The first is involved in the synthesis of citrate from pyruvic acid and the 4-carbon dicarboxylic acid. The other is required for the conversion of  $\alpha$ -ketoglutaric acid to succinic acid. Although it is known that cocarboxylase is essential for the oxidation of pyruvic acid, it is not yet known whether cocarboxylase is required for both of the decarboxylations postulated in the cycle. The evidence which we have presented demonstrates that the vitamin is essential for the synthesis of citric acid, because the excretion of this compound is low during the deficiency despite the presence of excess pyruvic acid, and because the rise in excretion noted when succinate is injected is considerably less in the thiamine-deficient animal than in the normal. It should also be emphasized that the decreased citrate excretion is independent of the inanition accompanying the deficiency, since fasted control animals show no decrease in citrate excretion.

The curative experiments demonstrate a consistent rise in citrate excretion for 4 to 6 days after therapy before maximum values are attained. Excretion then returns to normal, while growth and food consumption are still maintained. Direct correlation with food intake cannot be established, for food intake and growth rate reach a maximum within 48 to 72 hours after thiamine therapy, while the maximum citrate excretion is not reached until 2 to 3 days later. In addition, at the peak of excretion the citrate excreted is greater than the citrate intake.

Finally, the drop in citrate excretion occurs while the intake of the experimental ration is still high.

One would expect that, as a result of the excess pyruvate present in the animal during the deficiency, the maximum citrate excretion would occur immediately after vitamin therapy. That this is not true suggests that the mechanism for the synthesis of citric acid is not regenerated immediately after administration of the vitamin, but increases for several days before full activity is restored. While it is true that the disappearance of polyneuritic symptoms within a few hours following therapy implies the rapid restoration of the integrity of the nervous system, there is no reason to believe that all of the organs return to normal activity at the same rate. In support of this view, it may be pointed out that Engel and Phillips (37) have demonstrated pathological changes in the liver of deficient rats and chicks after the administration of the vitamin and the cure of the external symptoms. Several weeks on a normal ration were required for the restoration of the normal histological picture. It will also be recalled that, while the respiration of polyneuritic brain tissue *in vitro* may be restored by the addition of thiamine to the medium, no catatorulin effect can be observed with liver and muscle (38).

The reason for the rise in citrate excretion following thiamine therapy is thus far not clear. Similar rises occur as a result of the ingestion of alkali (23) or upon the injection of certain citrate precursors (28, 29). Neither of these phenomena offers a likely explanation of our results.

Either the tissue enzymes are disorganized as a result of the therapy, leading to more rapid formation of citrate than can normally be catabolized, or the mechanism for the catabolism of citrate as well as for its synthesis is affected by thiamine deficiency and is not restored at a rate equal to that of the synthetic mechanism. The former view would present a situation which is perhaps analogous to that observed by Engel and Phillips (37) with respect to liver fat; the latter may explain the temporary rise in citrate excretion during early thiamine deficiency.

#### SUMMARY

1. Studies upon the excretion of citric acid during thiamine deficiency indicate a decreased excretion of citrate during the

acute deficiency. This is independent of the inanition accompanying the deficiency.

2. Rats suffering from thiamine deficiency show a decreased ability to transform injected succinic acid into citric acid.

3. A 10-fold rise in citric acid excretion is observed within 4 to 6 days after thiamine is given to deficient rats. The level then drops slowly and normal values are restored in 10 to 14 days after the initiation of therapy.

4. The results are discussed with respect to the metabolism of citric acid and it is concluded that thiamine pyrophosphate is essential for the synthesis of endogenous citric acid from its precursors.

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## PROPERTIES OF TROPINE ESTERASE\*

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The extensive use of tropine esters in the medical field, the importance of these compounds for biological investigations, and the interest attending their use as substrates in enzyme reactions make a study of their enzymatic destruction desirable.

A survey of previously published researches concerning the enzymatic hydrolysis of tropine esters reveals that some data have been accumulated relating to the distribution of these enzymes in the animal body, but there is little information about the effects of various hydrogen ion concentrations, temperatures, enzyme-substrate affinities, and the specificity. The first three properties are to be considered in the present communication; and specificity, together with further proof that tropine esterase is an enzyme distinct from cholinesterase, is being studied in collaboration with Dr. Susi Glaubach, and will form the basis of a paper to follow.

Employing a biological method of assay for atropine, Lévy and Michel (1) showed that this substrate was increasingly destroyed by rabbit blood with increase of pH in the range 6.6 to 8.0; and, further, greater destruction occurred with an increase of temperature (5°, 38°, and 50° were those investigated). The greater hydrolysis at 50° compared to 38° is not in accord with the experiments recorded in Fig. 5.

Bernheim and Bernheim (2) have studied the activity-time relationships for the hydrolysis of atropine and homatropine by guinea pig liver, using a direct chemical estimation of the acid liberated. From the shape of their curves, one would conclude that the reactions are of a zero order.

\* Aided by a grant from the Sidney C. Keller Research Fund.



## EXPERIMENTAL

The disadvantages of biological methods for estimation of tropine esterase activity, both in regard to accuracy and technique, are obvious. Bernheim and Bernheim (2) are the only investigators who employed a direct chemical determination of the acid liberated to follow the enzymatic hydrolysis. They employed the manometric procedure, using the Warburg apparatus in a fashion similar to that evolved for choline ester splitting. Other chemical methods have been used, such as the nephelometric analysis of unhydrolyzed atropine (3), but they cannot compare in ease and precision with methods based on the determination of free acid.

Atropine sulfate, Merck, was used as substrate. The source of enzyme chosen was rabbit serum, since it was readily available and maintained a practically constant activity for several months when stored over chloroform in the refrigerator. As is well known, the serum from many rabbits does not contain tropine esterase; hence a number of sera had to be tested before an active sample was obtained.

Before studies of pH and temperature effects were undertaken, it was deemed advisable to establish the activity-substrate concentration relationship, and as a preliminary to this it was necessary to find the concentration of serum most suitable.

The manometric procedure, used by Bernheim and Bernheim (2), was employed for these studies on activity-enzyme concentration and activity-substrate concentration relationships. There were a number of variables which had to be arbitrarily fixed in the first experiments. Accordingly, the determination of the effect of enzyme concentration, first undertaken, was carried out at 30° in the bicarbonate-Ringer medium of pH 7.4; the total volume was 4 ml., containing a final concentration of 1 per cent atropine sulfate (1 ml. of enzyme solution, prepared by diluting serum with bicarbonate-Ringer's solution, was placed in the side arm of the Warburg vessel, and 3 ml. of the substrate dissolved in bicarbonate-Ringer's solution were pipetted into the main chamber). Manometer readings were taken every 30 minutes for 2 hours, and slopes of the linear activity-time curves thus obtained for four dilutions of serum were used as points to establish the curve given in Fig. 1. The hydrolyses observed were corrected

in each instance for non-enzymatic atropine splitting by control experiments in which 1 ml. of bicarbonate-Ringer's solution was substituted for the serum solution. Only corrected values were plotted.

From Fig. 1 it may be seen that the maximum concentration of serum which still gives values on the linear portion of the curve is 2.5 per cent. Since the rate of hydrolysis of atropine is relatively

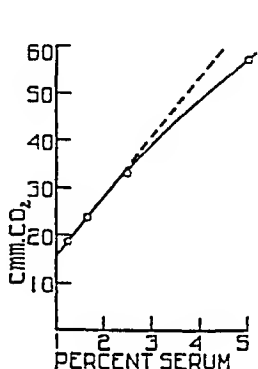


FIG. 1

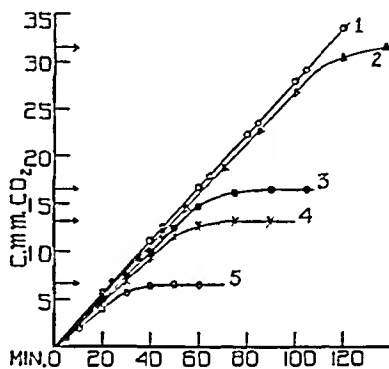


FIG. 2

FIG. 1. Tropine esterase activity as a function of serum concentration. The broken line is the extrapolation of the linear portion of the curve. The points represent hydrolysis of 1 per cent atropine sulfate after 2 hours at 30° and pH 7.4.

FIG. 2. The course of enzymatic hydrolysis of atropine in various concentrations at 30° and pH 7.4 by 2.5 per cent serum. The arrows on the vertical axis indicate the quantity of CO<sub>2</sub> equivalent to 100 per cent hydrolysis of the four lowest substrate concentrations. Curve 1 represents atropine concentrations of 0.00300 M (□) and 0.00150 M (●); Curve 2, 0.00074 M; Curve 3, 0.00037 M; Curve 4, 0.00030 M; and Curve 5, 0.00015 M.

slow, this maximum serum concentration was used for all subsequent experiments.

In Fig. 2 the activity-time curves for various substrate concentrations are given, and in order to apply these data to the calculation of the affinity between enzyme and substrate according to the Michaelis-Menten theory, as has been done previously for other esterases (4, 5), Table I was compiled from which the points shown in Fig. 3 were obtained. The linear portions of the curves for the three lowest substrate concentrations were extrapolated to 100 minutes. The curve in Fig. 3 represents the

theoretical activity- $pS$  relationship ( $pS$  = negative logarithm of substrate concentration) for a Michaelis constant ( $K_s$ ) of  $6 \times 10^{-5}$ ; the agreement between this curve and the experimentally obtained points is apparent.

The manometric procedure used above is not applicable to measurements of the effect of pH on activity, since the pH range over which it may be used is small. For this reason continuous titration in a buffer-free medium, with a glass electrode to indicate pH, was employed in a fashion similar to that used previously for pH studies on cholinesterase (5).

In the present instance constant temperature was maintained by immersing the reaction flask in the water of a thermostat.

TABLE I  
*Data for Calculation of Affinity between Enzyme and Substrate*

Concentration of atropine sulfate		CO <sub>2</sub> in 100 min.	A factor, $3.48 \times \text{c.mm.}$ CO <sub>2</sub> in 100 min.
<i>per cent</i>	<i>M</i>	<i>c.mm.</i>	
0.250	0.00740	28.5	99.3
0.100	0.00300	28.0	97.5
0.050	0.00150	28.0	97.5
0.025	0.00074	26.6	92.8
0.0125	0.00037	25.0	87.0
0.010	0.00030	23.5	81.8
0.005	0.00015	20.3	70.8

The reaction vessel was a 75 ml. round bottom flask having four necks which protruded above the level of the water in the thermostat. The glass and calomel electrodes fitted into two of the necks, and the other two contained 1-hole rubber stoppers fitted with glass tubes drawn out to fine tips. One of these glass tubes served as a gas jet through which nitrogen was passed. (The nitrogen was previously saturated with water vapor by being bubbled through a cylinder of water immersed in the same thermostat.) The other glass tube was connected to a microburette so that standard alkali could be added to the reaction mixture to neutralize the acid as fast as it was formed by the hydrolysis. Both glass tips were placed above the surface of the reaction mixture in the flask.

Bubbling the nitrogen through the liquid for purposes of mixing

the added alkali was not found to be feasible, because it produced a great deal of foaming. However, agitation was finally effected by applying motion to the flask. The flask and burette were both held in position by burette clamps fastened to a short vertical metal rod. This rod, in turn, was fastened by a clamp to a horizontal rod extending over the thermostat, and the horizontal rod was supported at each end by a clamp fastened to a ring-stand.

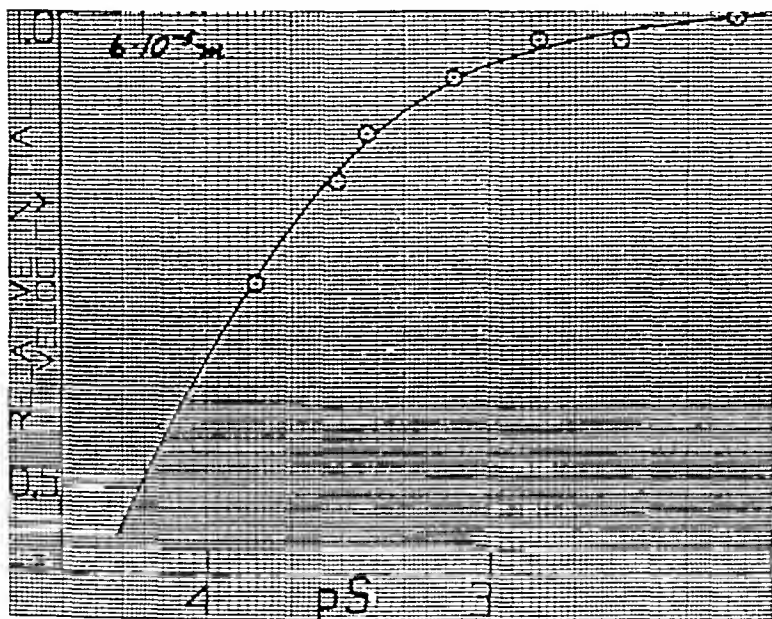


FIG. 3. Activity- $pS$  curve for atropine hydrolysis by rabbit serum. The point of inflection occurs at a substrate concentration of  $6 \times 10^{-5} M$  and is numerically equal to the Michaelis constant.

By loosening the screws that would fix the horizontal rod, the latter could be rotated. Agitation was produced by merely moving the vertical rod back and forth, which imparted an angular motion to the horizontal rod, the clamp fasteners fixed to the ring-stands forming bearings.

By the method above, the formation of acid could be followed in a mixture consisting only of enzyme and substrate, under conditions of practically constant  $pH$  and temperature, and in a

nitrogen atmosphere which eliminated the effects of carbon dioxide in the air. The lead connecting the glass electrode to the potentiometer and the glass electrode itself were shielded. The burette was divided into 0.01 ml. graduations, and readings were taken to 0.001 ml. Standard 0.01 N or 0.02 N NaOH was used, depending upon the speed of the reaction.

The final reaction volume in every case was 20 ml. 0.5 ml. of serum was employed, giving the 2.5 per cent concentration already mentioned as being the most desirable. The final concentration of atropine was 0.25 per cent which is the lowest concentration

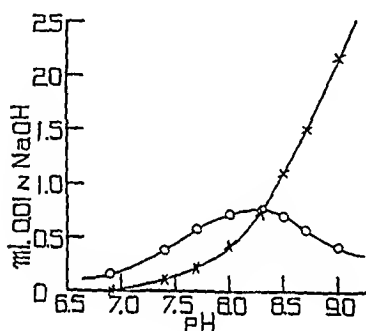


FIG. 4

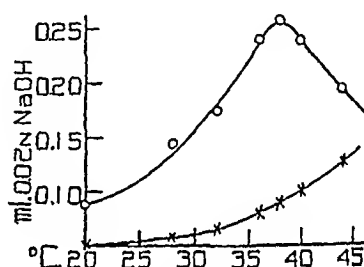


FIG. 5

FIG. 4. Tropine esterase activity as a function of pH.  $\odot$  represents enzymatic hydrolysis of 0.25 per cent atropine sulfate at 40° after 100 minutes by 2.5 per cent serum;  $\times$ , the corresponding non-enzymatic hydrolysis.

FIG. 5. Tropine esterase activity as a function of temperature.  $\odot$  indicates enzymatic hydrolysis of 0.25 per cent atropine sulfate at a pH of 8.4 after 30 minutes by 2.5 per cent serum;  $\times$ , the corresponding non-enzymatic hydrolysis.

of this substrate giving the maximum rate of hydrolysis (Fig. 3). Control experiments without serum were performed in each case, and the corrections applied. It should be mentioned here that, although the non-enzymatic splitting of atropine in low concentrations is negligible as Bernheim and Bernheim (2) have pointed out, still in concentrations of the order of those found most suitable (0.25 per cent), and for all higher concentrations, this factor is appreciable and necessitates control experiments. Furthermore, non-enzymatic hydrolysis must be considered in cases in which higher pH values and temperatures are employed (Figs. 4 and 5).

The results of the pH measurements carried out in this manner

are given in Fig. 4. The experiments were conducted at 40°, since this is in the region of the temperature optimum for other esterases. The degree of hydrolysis was a linear function of time for the experimental period.

Finally, measurements of temperature effects were made by the technique used for the pH studies (Fig. 5).

#### DISCUSSION

As Bernheim and Bernheim (2) found for tropine ester hydrolysis by guinea pig liver, the course of the enzymatic scission produced by rabbit serum may be seen to be a linear, or zero molecular, reaction (Fig. 2). Here, as in the case of liver esterase acting upon simple esters (4), the linearity is due to the high affinity between enzyme and substrate.

The Michaelis constant,  $6 \times 10^{-5}$ , derived from Fig. 3, indicates an unusually high affinity between atropine and tropine esterase, since the magnitude of the constant is inversely proportional to the affinity. In fact, the affinity in this case is the highest recorded for any of the esterases. It is approximately 18 times that between cholinesterase and acetylcholine ( $K_s = 0.0011$ ) (5), and 33 times that between liver esterase and methyl butyrate ( $K_s = 0.0020$ ) (4). Reference to the table of constants in Haldane's book (6) shows that not one of the values given, regardless of the nature of the enzyme and substrate, is as low as that found for the tropine esterase-atropine combination. The value most nearly approaching it is 0.00018 found by Weber and Ammon for pig liver acting upon methyl *d*-mandelate.

As in the case of cholinesterase, this enzyme showed no inhibition by high substrate concentrations, the same rate of hydrolysis being observed in the presence of 1 per cent atropine sulfate as in 0.25 per cent. In this regard a difference exists between these two enzymes and liver esterase, which is inhibited by excess substrate.<sup>1</sup>

The activity-pH relationship for tropine esterase (Fig. 4) is very much the same as that previously observed for cholinesterase (5, 8), the maximum falling in the range 8.1 to 8.4 for the former,

<sup>1</sup> After this paper was submitted for publication, Alles and Hawes (7) reported that human blood cells contain a cholinesterase which is inhibited by excess substrate.

and 8.4 to 8.5 for the latter. Esterases acting upon simple esters have their pH optima in the same regions, although values ranging from 7.0 to 8.8 have been reported from various laboratories.

The activity of tropine esterase as a function of temperature (Fig. 5) is also similar to that found for cholinesterase (9), the optimum occurring at 38° in the case of the former and at 40° in the latter. Temperature coefficients (the ratio of the activity at a given temperature to that 10° lower) may be calculated from the curve in Fig. 5. Thus the value is 2.00 for the range 25-35°, and 1.90 for 28-38°. These values are higher than those reported for cholinesterase and ethyl butyrase (9).

From Fig. 5 one can obtain velocity constants of the enzymatic reaction at different temperatures, and these, in turn, may be used to calculate temperature constants according to the formula

$$A = \frac{\log k_2 - \log k_1}{0.4343} R \frac{T_2 T_1}{T_2 - T_1}$$

where  $k_1$  and  $k_2$  are velocity constants referring to absolute temperatures  $T_1$  and  $T_2$ , and  $R$  is the gas constant.  $k = \text{c.mm. of CO}_2 \text{ per minute} = 0.135/30 \text{ or } 0.00450 \text{ for } 28^\circ$ , and  $0.257/30 \text{ or } 0.00857 \text{ for } 38^\circ$ . Hence  $A = (\log 0.00857 - \log 0.00450) (93,611/10) (4.581) = 12,000 \text{ calories}$ . This value is more than twice that obtained for cholinesterase (9).

#### SUMMARY

Studies by chemical methods were carried out concerning the effects of enzyme and substrate concentrations, pH, and temperature upon the enzymatic hydrolysis of atropine by rabbit serum.

The hydrolyses were zero molecular reactions, and optimum conditions were found to be pH 8.1 to 8.4 and 38°. The highest serum concentration in accordance with the linear relation between enzyme concentration and activity was found to be 2.5 per cent. The lowest substrate concentration giving the maximum rate of enzymatic hydrolysis was shown to be 0.25 per cent atropine sulfate.

The Michaelis constant was determined, and the value obtained ( $6 \times 10^{-5}$ ) indicated an unusually great affinity between

enzyme and substrate. No excess substrate inhibition was observed.

The temperature coefficient and constant were calculated.

The author wishes to thank Mr. Sidney Morett for his technical assistance.

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# DETERMINATION OF PROLINE IN MIXTURES CONTAINING *l*- AND *dl*-PROLINE

## THE PROLINE CONTENT OF GELATIN

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In a recent communication (1) there was described a method for the determination of amino acids, based on the law of the constancy of the solubility product. With the aid of this method, and with ammonium rhodanilate as reagent, an *l*-proline content of 17.5 per cent was found for various gelatins and for tendon collagen. It was doubtful whether this figure represented the total proline content of the proteins investigated; for, if partial racemization of proline occurred during the hydrolysis of the proteins with boiling HCl, the presence of the *d* isomer might not have been detected. In order to clarify this point, studies were undertaken of the behavior of mixtures of *l*- and *d*-proline when precipitated with ammonium rhodanilate under the conditions employed in the estimation of proline by the solubility method.

It was found that, from a solution containing equimolecular amounts of *l*- and *d*-proline, there was precipitated a *dl*-rhodanilate<sup>1</sup> which was found to be considerably less soluble, in the methanol-water solvent employed, than is *l*-proline rhodanilate. The solubility product constant of *l*-proline rhodanilate is  $9.2 \times 10^{-6}$ , whereas the constant for *dl*-proline rhodanilate is  $3.1 \times 10^{-6}$ . It follows that the latter precipitate is a racemic compound, and not an inactive mixture of *l*- and *d*-proline rhodanilates.

When mixtures of *l*- and *dl*-proline were dissolved in aqueous HCl and a methanol solution of ammonium rhodanilate was added, the precipitates obtained consisted of mixtures of *l*-proline

<sup>1</sup> All the experiments with proline rhodanilate discussed in this paper were performed at 0°.

rhodanilate and its racemate. A comparison of Columns 3 and 4 of Table I reveals that the relative proportion of *l*- and *dl*-proline found, as rhodanilates, in the precipitate is in each case about the same as in the corresponding original mixture. Even when *dl*-proline comprises 90 per cent of the original mixture, no marked fractionation occurs in the course of the precipitation of the rhodanilates. It appears, therefore, that the law of the constancy of the solubility product is not the sole factor governing the

TABLE I

*Precipitation of Mixtures of l- and dl-Proline with Ammonium Rhodanilate\**

Proline employed (1)	Proline rhodanilate pptd.† (2)	<i>l</i> -Proline rhodanilate in ppt. (3)	<i>l</i> -Proline in original mixture‡ (4)
<i>mole</i>	<i>mole</i>	<i>per cent</i>	<i>per cent</i>
0.00386 <i>l</i> - + 0.00021 <i>dl</i> -	0.00337	97.8	95.8
0.00386 <i>l</i> - + 0.00042 <i>dl</i> -	0.00347	93.9	90.2
0.00386 <i>l</i> - + 0.00063 <i>dl</i> -	0.00359	88.7	86.2
0.00313 <i>l</i> - + 0.00090 <i>dl</i> -	0.00341	74.4	77.7
0.00044 <i>l</i> - + 0.00361 <i>dl</i> -	0.00363	10.6	10.9

\* For the experimental procedure, volume, and composition of solvent, etc., see the text.

† The amount of ammonium rhodanilate added was, in all cases, about 120 per cent of the theoretical quantity necessary to precipitate all the proline present.

‡ Calculated from Column 1.

equilibrium in this case. The data suggest that *dl*- and *l*-proline rhodanilates form solid solutions in all proportions.

Further indication of the presence of solid solutions is afforded by the data in Table II. Four mixtures of *l*- and *dl*-proline rhodanilate, of the composition indicated in Column 1, were dissolved in methanol and precipitated by the addition of aqueous HCl. The solid phase obtained was analyzed polarimetrically for its content of *l*- and *dl*-proline rhodanilate as described in a previous communication (1). The resulting data on the composition of the solid phase at equilibrium are given in Column 2,

and its solubility in Column 3. The solubilities of pure *dl*- and *l*-proline rhodanilates were measured under the same conditions, and are represented by the first and last figures in Column 3. The values in Column 4 are the solubilities calculated for solid solutions of the *l* and *dl* salts. In this calculation, the solubility,  $S$ , of a solid solution in the liquid phase is considered to be  $S = M_l S_l + M_{dl} S_{dl}$ , where  $M_l$  and  $M_{dl}$  represent the mole fractions of the *l* and *dl* components in the solid phase at equilibrium, and  $S_l$  and  $S_{dl}$  are the solubilities of the pure *l* and *dl* components

TABLE II  
*Solubility of Solid Solutions of l- and dl-Proline Rhodanilates\**

Composition of mixture of rhodanilates employed†	Composition of solid phase at equilibrium	Solubility of solid phase at equilibrium	
		Found (3)	Calculated‡ (4)
(1)	(2)		
per cent	per cent	mg. per 350 cc.	mg. per 350 cc.
100 <i>dl</i> -	100 <i>dl</i> -	297	
80 " + 20 <i>l</i> -	79.5 <i>dl</i> - + 20.5 <i>l</i> -	329	344
60 " + 40 "	65.4 " + 34.6 "	373	377
40 " + 60 "	45.2 " + 54.8 "	428	424
20 " + 80 "	20.6 " + 79.4 "	489	481
100 <i>l</i>	100 <i>l</i> -	529	

\* For the experimental procedure, volume, and composition of solvent, etc., see the text.

† The amount of original mixture employed was, in all cases, about 2.0 gm.

‡ In calculating the solubilities in Column 4, the molecular weight of *dl*-proline rhodanilate is assumed to be the same as that of *l*-proline rhodanilate.

in the water-methanol solvent. Meyer (2) has found that this simple equation, which follows from Raoult's law, best fits the solubility data yielded by solid solutions of two binary electrolytes having a common ion. From the data obtained here for the proline rhodanilates, it is observed that the solubilities calculated in this manner are in close agreement with the experimental values.

A consideration of the foregoing experiments with known mixtures indicates that it should be possible readily to detect the presence of *d*-proline in a protein hydrolysate. This premise was checked on a gelatin hydrolysate in the following manner.

To a hydrolysate of 2.59 gm. of water-free gelatin (corresponding to about 450 mg. of *l*-proline) there were added 34 mg. of *dl*-proline (corresponding to about 7.1 per cent of the total proline content). The proline rhodanilate precipitate obtained in the usual manner was analyzed polarimetrically and found to contain 91.8 per cent of *l*-proline rhodanilate and 8.2 per cent of *dl*-proline rhodanilate. The proline rhodanilate obtained from another sample of the same hydrolysate, to which no *dl*-proline had been added, was found to contain 99.1 per cent of pure *l*-proline rhodanilate.

From the above information it may be concluded that in the hydrolysates of various gelatins and of collagen that have been analyzed recently (1), the *d* isomer content could not have been more than about 1.5 per cent of the total proline. All the precipitates of proline rhodanilate obtained in the course of these earlier analytical procedures were examined polarimetrically, and in all cases a rotation corresponding to 97 to 100 per cent of *l*-proline rhodanilate was observed. An appreciable amount of the *d* or the *dl* form in the hydrolysate should have manifested itself in a lower rotation of the rhodanilates.

Although it has been shown that the hydrolysates investigated earlier contained only a negligible amount of *d*- or *dl*-proline, it will be demonstrated later that during prolonged hydrolysis of gelatin with boiling HCl appreciable racemization of proline occurs. It is desirable to ascertain, therefore, whether it is possible, by the solubility method, to determine the total proline content of a mixture containing *l*- and *dl*-proline. To achieve such a determination, it is a necessary condition that the solubility product of the proline rhodanilate be a constant, despite the stereochemical inhomogeneity of the proline.

The results of two determinations on known mixtures, the one containing 22.3 per cent of *dl*-proline and 77.7 per cent of *l*-proline, the other containing 89.1 per cent of *dl*-proline and 10.9 per cent of *l*-proline, are recorded in Table III. A consideration of the data in Table III indicates that the above condition is fulfilled, and that a satisfactory determination of the total proline content of such mixtures is possible. The proline content found (Column 2) was calculated simply from the weights of the proline rhodanilate precipitates obtained in the course of the determination, without regard to their stereochemical character.

When determinations of the total proline content of proteins are performed, it is important to ascertain the optimum conditions of hydrolysis and to know whether proline is destroyed or racemized in the course of prolonged hydrolysis. In order to check this point the proline content of a gelatin hydrolysate was determined after 3 hours of hydrolysis with boiling concentrated HCl.<sup>2</sup> The total proline content was found to be 17.0 and 17.3 per cent. The proline rhodanilate precipitates obtained in these determinations exhibited the rotation of the pure *l* form. These results are in close agreement with the value, 17.5 per cent, previously obtained after 8 hours of hydrolysis.

TABLE III

*Determination of Total Proline Content of Solutions of l- and dl-Proline*

Proline content of solutions employed	Total proline content found	Average recovery	Solubility product* constant	<i>l</i> -Proline rhodanilate content of ppts.
(1)	(2)	(3)	(4)	(5)
<i>mole</i>	<i>mole</i>	<i>per cent</i>	$\times 10^{-1}$	<i>per cent</i>
0.00313 <i>l</i> - +	0.00403		8.5	73.5
0.00090 <i>dl</i> -	0.00391	97.5	8.3	73.9
	0.00382		9.4	74.4
0.00361 <i>dl</i> - +	0.00424	104	3.8	7.9
0.00044 <i>l</i> -			3.2	10.6

\* The solubility products are, in each case, the product of the concentrations of the proline and the rhodanilic acid remaining in the solutions. The concentration of the proline is considered to be the sum of the concentrations of the stereoisomers. Cf. also Bergmann and Stein ((1) p. 219, foot-note 1).

On the other hand, when gelatin was hydrolyzed under similar conditions for 48 hours and proline determined as before, each of the proline rhodanilate precipitates obtained gave the theoretical C and H values for proline rhodanilate and contained 86.6 per cent of *l*-proline rhodanilate and 13.4 per cent of *dl*-proline rhodanilate. The total proline content of the hydrolysate, however, was found to be 17.1 per cent, in good agreement with the values obtained after 3 and 8 hours hydrolysis. Apparently a portion of the

<sup>2</sup> The ratio  $\text{NH}_2\text{-N}$  to total N was 0.63. After the hydrolysate was cleared by the CuS procedure (1), the ratio  $\text{NH}_2\text{-N}$  to total N had risen to 0.66.

proline was racemized during 48 hours hydrolysis, but no appreciable amount was destroyed.

Experiments of this type naturally do not indicate whether some proline is destroyed in the peptide stage during the first 3 hours of the hydrolysis. In the absence of evidence for destruction of proline in the peptide stage, the value 17.5 per cent ( $\pm 0.5$  per cent) may be regarded as representing the proline content of gelatin and tendon collagen.

#### EXPERIMENTAL

*Precipitation of Mixtures of l- and dl-Proline with Ammonium Rhodanilate*—In order to demonstrate the way in which the data in Table I were obtained, the first experiment in the table may be described. 555 mg. of *l*-proline and 30 mg. of *dl*-proline were dissolved in water, 35 cc. of *N* HCl were added, and the volume was made up to 250 cc. The solution was cooled, and a 200 cc. aliquot (containing 0.00386 mole of *l*-proline and 0.00021 mole of *dl*-proline) was added to 2.505 gm. of ammonium rhodanilate dissolved in 130 cc. of cold methanol. The solution was agitated at 0°, in a tightly stoppered flask, for 48 hours. The precipitate was collected at 0° on weighed crucibles with sintered glass filter plates, and dried over CaCl<sub>2</sub> and KOH in a desiccator to constant weight. The precipitate was analyzed polarimetrically in the manner already described.<sup>3</sup>

*Solubility of Solid Solutions of l- and dl-Proline Rhodanilates*—2.000 gm. samples of the mixtures of the composition indicated in Column 1, Table II, were dissolved in 130 cc. of cold methanol, and 200 cc. of ice-cold water containing 25 cc. of *N* HCl were added to each. The mixtures were agitated at 0° for 48 hours, and the precipitates were filtered, dried, weighed, and analyzed polarimetrically. The results of the polarimetric analysis are reported in Column 2, Table II. The solubilities, reported in Column 3, Table II, were determined as the difference in weight between the mixtures employed and the precipitates recovered.

<sup>3</sup> In our earlier communication (1), the equation for calculating the *l*-proline content of a sample of proline rhodanilate subjected to polarimetric analysis was given erroneously. The equation should read  $p = (\alpha \times S \times 57)/(-1.52^\circ \times 3.19)$  ( $p$  represents proline in mg.,  $\alpha$  observed rotation, and  $S$  total weight of solvents in gm.).

The precipitate containing 79.5 per cent of *dl*-proline and 20.5 per cent of *l*-proline and the one containing 20.6 per cent of *dl*-proline and 79.4 per cent of *l*-proline were analyzed for their carbon and hydrogen content.

$(C_{16}H_{14}N_4S_4Cr) \cdot (C_5H_{10}O_2N) \cdot H_2O$	Calculated.	C 41.7, H 4.3
604.6	Found.	" 41.8, " 4.3
	"	" 41.7, " 4.4

*Quantitative Determination of Total Proline Content of Mixtures of l- and dl-Proline*—515 mg. of *dl*-proline, 1.800 gm. of *l*-proline, and 140 cc. of *N* HCl were made up to 1 liter with water. The solution was cooled, and three 200 cc. aliquots, each containing 0.00403 mole of total proline, were added to 1.800, 2.202, and 2.603 gm. samples of ammonium rhodanilate each dissolved in 130 cc. of cold methanol. After 48 hours agitation at 0°, the precipitates were collected, dried, weighed, and analyzed polarimetrically. The precipitates weighed 1.675, 1.929, and 2.062 gm. and contained 73.5, 73.9, and 74.4 per cent of *l*-proline rhodanilate and 26.5, 26.1, and 25.6 per cent of *dl*-proline rhodanilate respectively. The three calculated values for the total proline content of the aliquots taken were 0.00403, 0.00391, and 0.00382 mole; average 0.00392 mole, corresponding to 97.5 per cent of the theory.

The analysis of the other mixture mentioned in the text was performed in the same manner.

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## CREATINE FORMATION IN LIVER AND IN KIDNEY\*

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We reported recently (1) the formation of creatine from glycocyamine by rat liver slices; and that 40 to 50 per cent more creatine was formed when methionine was added with the glycocyamine to the Ringer's solution in which the slices were immersed. Among some thirty odd amino acids, methylated amines, a methylated purine, and betaine only methionine gave this increased rate of methylation. The rate of creatine formation under these conditions is sufficient, if it is of the same order of magnitude *in vivo*, to make good the entire loss as urinary creatinine.

If the methylation of glycocyamine in the liver is important both as a mechanism and as a site of creatine formation in animals, one may expect to find it in the livers of most other animals. We accordingly examined the livers of a number of animals for their ability to carry out this reaction. At the same time the possibility of its occurring in their kidneys was investigated also.

The findings in this survey, summarized in Table I, were that glycocyamine can be methylated by the liver of every animal examined; the stimulating effect of methionine observed with rat liver was found with some but not all the other animals; the kidney appears to be unimportant (except in the pigeon) for the methylation of glycocyamine without or with methionine.

The experimental and analytical procedure was the same as was described in our previous communication, except that the experiments with frog liver and kidney were carried out at 25° instead

\* Presented before the meeting of the American Society of Biological Chemists at New Orleans, March 13-16, 1940 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, 133, p. xv (1940)).

of 38°. All the creatine figures given represent differences in chromogenic material in the Jaffe reagent before and after digestion with the NC bacteria of Miller and Dubos (4).

Baker and Miller (3) have reported that creatine is formed from unidentified precursors in rat liver and kidney (slices and mash). Our findings on this point are that small amounts of creatine (beyond the experimental error) were formed from pre-

TABLE I

*Formation of Creatine in Liver and Kidney Slices*

Temperature 38°; glucose-Ringer's solution. The creatine is given as mg. per 100 gm. of wet weight of tissue.

Animal	Creatine in liver				Creatine in kidney			
	In- initially present	After 4 hrs. incubation			In- initially present	After 4 hrs. incubation		
		In Ringer's solution alone	With 6.25 mg. per cent glyco- cyam- ine	With 6.25 mg. per cent glyco- cyamine and 40 mg. per cent dl-methi- onine		In Ringer's solution alone	With 6.25 mg. per cent glyco- cyam- ine	With 6.25 mg. per cent glyco- cyamine and 40 mg. per cent dl-methi- onine
Cat.....	12.0	12.2	21.1	23.2	1.9	2.5	2.4	2.3
Dog.....	2.9	2.9	3.5	10.3	17	26	26	26
Guinea pig .....	1.7	3.4	12.4	12.1	3.0	4.5	8.5	6.6
Frog (25°).....	2.2	2.2	4.8	8.4	36	44	47	45
Pigeon.....	1.0	2.0	11.8	16.0	10.8	10.7	17.9	22.3
Rabbit.....	5.8	5.7	26.4	12.0	9.4	15.2	15.0	15.4
Rat.....	1.4	2.8	20.6	28.4	10.8	18.6	16.3	18.2

The initial values are lower than those given by Baker and Miller (2, 3) for rat liver and kidney. In our procedure the slices are washed in a large volume of Ringer's solution for at least 3 minutes before they are transferred to the reaction vessels or submitted to analysis. Preformed creatine undoubtedly diffuses out of the slices during this washing.

existing precursors in the liver slices of the guinea pig, pigeon, and rat. Kidney slices gave significant increases in every case except in the pigeon.

After the cell structure of rat liver and kidney was destroyed, no creatine was formed from preexisting precursors nor from glyco-cyamine without or with methionine. The homogenizing procedure of Potter and Elvehjem (5) was used to disrupt the cells. It would seem that the creatine formed by chopped rat liver and

kidney from preexisting precursors (Baker and Miller (3)) must be ascribed to the intact cells in the mash rather than to a liberated enzyme.

It is difficult to account for the large (absolute) amounts of creatine formed from preexisting precursors in the kidney slices in every case except in the pigeon, in view of the inability of cat, dog, rabbit, and rat kidney slices to methylate added glyco-cyamine, even in the presence of added methionine. The one animal which was negative in this respect—the pigeon—was the only animal whose kidney gave a positive effect with glyco-cyamine and a further increase with methionine.

When the Ringer's solution contained glyco-cyamine, there were large increases in creatine with the liver slices of every animal except the dog. In the interpretation of this negative effect in the case of the dog the positive effect observed when methionine was added with the glyco-cyamine must be taken into account. One possible interpretation is that the preexisting concentration of the glyco-cyamine-methylating agent is very low in dog liver—lower than in the livers of any of the other animals investigated.

With kidney slices the added glyco-cyamine was not methylated or was methylated only to an insignificant extent in every case except in the guinea pig and pigeon.

Bodansky (6) found an increase in the creatine content of rat kidney after glyco-cyamine was fed. Our interpretation of this increase was that it arose from temporary storage in the kidney of creatine synthesized in the liver from the glyco-cyamine fed (1). This interpretation is based on the finding that rat kidney slices do not methylate glyco-cyamine under conditions in which methylation readily occurs in liver. Bodansky concurs in this interpretation (personal communication).

Methionine accelerated the methylation of glyco-cyamine by liver slices of the dog, frog, pigeon, and rat. This stimulation was not observed with the cat, guinea pig, and rabbit. Two possible reasons for this failure are that the livers of these animals contained a plethora of the methylating agent, or that the relatively high concentration of the unnatural isomer in the *dl*-methionine used was inhibitory (as in the rabbit liver slices).

The pigeon was the only animal whose kidney slices were stimulated by methionine in their methylation of glyco-cyamine.

The pigeon is exceptional as far as creatine formation is concerned in that its kidney appears to share this function with its liver and resembles in this respect the livers rather than the kidneys of the other animals.

The negative effect of methionine with the kidney slices of all the animals tried except the pigeon is a little unexpected. Oxidative deamination is more active in kidney than in liver slices (7); and after oxidative deamination the lability of the S-methyl group is increased (8). It might have been expected therefore that the methylation of glycocyamine would be more active in kidney than in liver. This is not the case.

Our finding in this survey, that methylation of glycocyamine is an active function of the livers of all the animals examined, strengthens the conclusion that creatine is formed normally in the liver by the methylation of glycocyamine. It would be surprising if this is a fortuitous coincidence of little or no physiological significance. Other mechanisms and other sites of creatine formation are, of course, not excluded by these findings.

The significance of methionine here is less clear. Our findings suggest that it is the methylating agent of glycocyamine in liver or its precursor. We are hesitant at present to accept methionine itself as the methylating agent because its effect is small considering the concentration used. It may be that the actual methylating agent is the product of the oxidative deamination of methionine. This and other possibilities are now under investigation.

Du Vigneaud and coworkers (9) have shown that the presence of choline in the diet enables the animal to utilize homocystine or homocysteine in place of methionine. They have suggested that choline enables the body to methylate the sulfhydryl group of homocysteine by furnishing the required methyl group, and further that methionine may furnish the methyl groups of choline. The reality of the latter relationship is now established by direct proof recently presented by du Vigneaud.<sup>1</sup>

Our observations suggest that the S-methyl group of methionine may participate also in the formation of the N-methyl group of creatine. In this connection and in view of our findings with dog liver slices it is interesting that Stekol and Schmidt (10) found

<sup>1</sup> Verbal communication at the meeting of the American Society of Biological Chemists at New Orleans, March 13-16, 1940.

in the dog an increase in urinary creatinine after *dl*-methionine was fed, and none after glycine, glutamic acid, or *l*-pyrrolidonecarboxylic acid.

#### SUMMARY

1. A survey was made of the possibility of creatine formation *in vitro* by slices of the livers and kidneys of the cat, dog, guinea pig, frog, pigeon, rabbit, and rat.

2. Evidence of the existence of small amounts of creatine precursors in the livers of the guinea pig, pigeon, and rat, and in the kidneys of all of the above animals except the pigeon was found.

3. This finding appears to be much less significant quantitatively than the much more active glycocyamine-methylating function of the liver slices of all animals investigated. Only guinea pig and pigeon kidney slices possessed this ability.

4. The above findings are interpreted as indicating that creatine normally is formed by the methylation of glycocyamine in the liver.

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# THE ACTION OF CYSTEINE OR CYANIDE UPON GONADOTROPIC EXTRACTS

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Fraenkel-Conrat, Simpson, and Evans (1) have recently reported that hypophyseal gonadotropic hormones may be completely inactivated by cysteine in mildly alkaline solution, whereas prolan, the principle from human pregnancy urine, shows no loss of potency under the same conditions; they have concluded that the disulfide bonds are essential for the activity of the hormones of pituitary origin and are non-essential or absent in the hormones of placental origin. In Foot-note 2 to their paper the statement is made that follutein (prolan) showed a slight decrease in potency.<sup>1</sup> It is also stated as curious and unexplained that when thymol was used as a preservative inactivation of prolان occurred but not when butanol was used. In the assay procedure used by these workers the establishment of a minimum effective dose by a positive response in two out of three rats is employed. Measures of variability are not given.

In our first chemical studies on the pituitary gonadotropic hormone (2) no detectable loss of activity occurred upon short exposures to nascent hydrogen, sulfur dioxide, hydrogen sulfide, ferrous sulfate, or hydrogen cyanide. Prolan (3) withstood the action of cyanide for 1 hour at pH 6.5. Unpublished data for prolان indicate less than 25 per cent inactivation upon exposure to nascent hydrogen (magnesium powder in dilute acetic acid). Because of these essentially negative results and the data of Fraenkel-Conrat *et al.*, which in our opinion are conflicting, the question of the reduction of gonadotropic hormones has been reopened.

<sup>1</sup> According to our interpretation of the data, this amounts to 70 per cent inactivation by cysteine.



## 642 Cysteine and Cyanide Action on Hormones

In this paper we report on (a) the action of cysteine upon the pituitary gonadotropic hormone, (b) the action of cysteine upon prolactin, and (c) the action of cyanide on both these hormones.

TABLE I  
*Action of Cysteine upon Pituitary Gonadotropic Hormone*

Ex-periment No.	Total dose of hormone per rat	Agent used to delay resorption	No. of injections per day	Treatment of hormone	Ovarian weight, average	No. of rats used to obtain average ovarian weight	Recovery of hormone
	mg.				mg.		per cent
I	1	None	5	Control	50	4	
	1	Tannic acid	1	" tannic acid after 12 hrs.	69	4	
	1	None	5	10 mg. powder, 10 cc. H <sub>2</sub> O, 200 mg. NaHCO <sub>3</sub> , 40 mg. cysteine HCl, 12 hrs.	26	4	40
	1	Tannic acid	1	Same cysteine treatment, then tannic acid pptn.	23	4	40
	2	None	5	6 mg. powder, 6 cc. H <sub>2</sub> O, 50 mg. NaHCO <sub>3</sub> , 12 mg. cysteine HCl, 24 hrs.	55	4	50
	2	"	5	6 mg. powder, 6 cc. H <sub>2</sub> O, 50 mg. NaOAc, 12 mg. cysteine HCl, 24 hrs.	43	4	50
II	1	Tannic acid	1	12.5 mg. powder, 6 cc. H <sub>2</sub> O, 120 mg. butanol, 650 mg. NaHCO <sub>3</sub> , 48 hrs., then HOAc and tannic acid pptn.	63	6	
	0.2	" "	1	Same treatment	12*	6	
	2	" "	1	12.5 mg. powder, 6 cc. H <sub>2</sub> O, 120 mg. butanol, 650 mg. NaHCO <sub>3</sub> , 125 mg. cysteine HCl, 48 hrs., then HOAc and tannic acid pptn.	12†	6	<10

\* 73 mg. uterine weight.

† 23 mg. uterine weight.

In view of the work on insulin, concentration of reagents and reaction time have been considered, as they have been shown to govern the degree of inactivation.

## EXPERIMENTAL

The gonadotropic preparations of pituitary and urine of pregnancy were the same as those used in earlier studies (2, 3). The treatment of the material and the details of assay are given in Tables I to IV. The chemical reactions took place at room temperature. The reaction products were kept at 5°. In all cases 22 to 23 day-old female rats were used for assay, and a

TABLE II  
*Action of Cysteine upon Prolan (Urine of Pregnancy)*

Experiment No.	Treatment of hormone	Ovarian weight, average	No. of rats used to obtain average ovarian weight	Recovery of hormone
		mg.		per cent
III	None	33	5	
	8 mg. prolان, 8 cc. H <sub>2</sub> O, 80 mg. cysteine HCl, 400 mg. NaHCO <sub>3</sub> , 48 hrs.	24	5	35
	Same + 0.2 cc. human blood serum	22	5	27
IV	8 mg. prolان, 400 mg. NaHCO <sub>3</sub> , 8 mg. cresol, 8 cc. H <sub>2</sub> O, 48 hrs.	26 ± 3.2	6	
	8 mg. prolان, 400 mg. NaHCO <sub>3</sub> , 8 mg. cresol, 80 mg. cysteine HCl, 8 cc. H <sub>2</sub> O, 48 hrs.	16 ± 2.0	6	24
V	10 mg. prolان, 530 mg. NaHCO <sub>3</sub> , 85 mg. butanol, 5 cc. H <sub>2</sub> O, 48 hrs.	29 ± 3.3	6	
	Same + 100 mg. cysteine HCl	16 ± 1.3	6	17
	10 mg. prolان, 530 mg. NaHCO <sub>3</sub> , 100 mg. cysteine HCl, 5 cc. H <sub>2</sub> O, 48 hrs.	18 ± 1.2	6	26

In all experiments a total of 1 mg. of prolان, untreated or treated, was administered per rat in four doses, one dose per day.

simultaneous assay of untreated material or control material, subjected to the same pH and preservatives as the chemically treated material, was made in litter mates. In the assay procedures, ovarian weight increase is the objective measure. Data on the measures of variability and the relation of ovarian weight increase to the level of dosage have been previously described (3, 4).

The cysteine reduction took place in bicarbonate buffer solution, the pH of the reaction approximating 8.0. In this pH range

insulin is almost completely (98 per cent) inactivated by its own weight of cysteine hydrochloride in 3 hours; it is 75 per cent inactivated in 1 hour (5). Cyanide reduction took place at approximately pH 10.0. This pH was chosen as the one used in analytic procedures for complete cysteine reduction (6).

TABLE III  
*Action of Cyanide upon Pituitary Gonadotropic Hormone*

Experiment No.	Treatment of hormone	Ovarian weight, average	No. of rats used to obtain average ovarian weight	Recovery of hormone
		mg.		per cent
VI	No treatment except solution in H <sub>2</sub> O	89	8	Complete
	10 mg. powder, 8 cc. H <sub>2</sub> O, 40 mg. NaCN, 80 mg. NaHCO <sub>3</sub> , pH 10.5, 1 hr., then acid to HOAc	73	8	
	12 mg. powder, 5 cc. H <sub>2</sub> O, 20 mg. NaCN, 15 min., then acid to HOAc	110	8	
VII	10 mg. powder, 300 mg. NaHCO <sub>3</sub> , 80 mg. Na <sub>2</sub> CO <sub>3</sub> , 3 cc. H <sub>2</sub> O, pH 10, 24 hrs., then acid to HOAc	55	6	>40
	10 mg. powder, 300 mg. NaHCO <sub>3</sub> , 100 mg. NaCN, 3 cc. H <sub>2</sub> O, pH 10, 24 hrs.,* then acid to HOAc	29	6	
	10 mg. powder, 300 mg. NaHCO <sub>3</sub> , 100 mg. NaCN, 3 cc. H <sub>2</sub> O, pH 10, 1 hr.,* then acid to HOAc	56	6	

In all cases the solution was rendered acid to HOAc after treatment, and the hormone precipitated with excess tannic acid; the precipitate was taken to volume in isotonic saline. The total dose per rat was 1 mg. of original powder given in four doses on 4 days.

\* Positive test to nitroprusside.

### Results

In Experiment I, Table I, which is concerned with the hypophyseal preparation, the assay of the cysteine-treated material gives the same result whether divided dosage or single dosage as the insoluble tannate is used, showing that the cysteine-treated material possesses the original resorption properties.<sup>2</sup> The

<sup>2</sup> In another paper (7), we have pointed out that the statements of Fraenkel-Conrat *et al.* in regard to the pituitary hormone were not proved, since the question of a change in resorption rate was not considered.

results of Experiments I and II, Table I, indicate that increasing amounts of cysteine and extension of the reaction time increase the inactivation.

A comparison of the experiments in Table I with those in Table II shows that under the same conditions of cysteine reduction more than 90 per cent inactivation of the pituitary hormone results, while from 63 to 85 per cent of inactivation of prolan

TABLE IV  
*Influence of Cyanide upon Prolan (Urine of Pregnancy)*

Experi- ment No.	Treatment of prolan	Ovarian weight, average	No. of rats used to obtain average ovarian weight	Re- covery of prolan
		mg.		per cent
VIII	10 mg. prolان, 300 mg. $\text{NaHCO}_3$ , 3 cc. $\text{H}_2\text{O}$ , 80 mg. $\text{Na}_2\text{CO}_3$ , 24 hrs., pH 10, then acid to HOAc, final volume 10 cc., pH 6.0	28	4	
	10 mg. prolان, 300 mg. $\text{NaHCO}_3$ , 3 cc. $\text{H}_2\text{O}$ , 100 mg. NaCN, 24 hrs., pH 10, then acid to HOAc, vacuum distillation, $0.1 \times \text{I}$ , $0.1 \times \text{Na}_2\text{S}_2\text{O}_3$ , final volume 10 cc., pH 6.0	21	4	40
IX	Control, same as Experiment VIII	34	5	
	NaCN-treated, same as Experiment VIII	23	5	33
X	Control, same as Experiment VIII, except 1 hr. at pH 10	$31 \pm 1.8$	6	
	NaCN-treated, same as Experiment VIII, except 1 hr. at pH 10	$35 \pm 2.4$	6	127

In all cases the total dose per rat was 1 mg. of prolان given in four doses on 4 successive days.

results whether or not butanol or cresol is used as preservative. In Experiment III, Table II, the addition of blood serum did not alter the response.

Experiments VI and VII, Table III, demonstrate that the pituitary hormone is able to withstand short exposures (1 hour) to cyanide in alkaline solution. 24 hours exposure produces some inactivation.

Experiments VIII, IX, and X, Table IV, show that the response of prolان to cyanide is like the response of the pituitary hormone.

In these experiments excess of HCN at the end of the reaction period was removed by vacuum distillation. The trace remaining was destroyed with 0.1 N iodine. The excess of iodine was destroyed with thiosulfate. That this procedure produces no inactivation is shown by the results of Experiment X.

#### DISCUSSION

According to Meyer (8)<sup>3</sup> prolan is sulfur-free. The fact that prolan can be partially inactivated by both cysteine and cyanide demonstrates that either the action of these reagents is by no means confined to disulfide reduction under the drastic conditions employed or that the purest prolan yet prepared is so crude that the sulfur content is too low for analysis. Prolan and the pituitary preparation withstand the action of cyanide under conditions in which sulfhydryl groups are produced in the pituitary preparation. In this laboratory (10), the study of the action of cyanide upon insulin showed that complete inactivation occurred in 18 hours, but that 30 to 60 per cent of the activity was recovered after 15 minutes exposure and that this partially inactivated insulin gave a positive nitroprusside test.<sup>4</sup> Wintersteiner (5), investigating the time curve of the reduction of the disulfide linkages of insulin by cysteine, and simultaneously observing the corresponding variation in physiologic activity, found that no proportionality exists between maximal reduction and physiologic activity, and that total inactivation occurs with the reduction of approximately one-third of the total sulfur. Both the pituitary and urinary gonadotropic preparations survive wholly or in part the action of cysteine or cyanide under conditions which completely inactivate insulin. The evidence taken as a whole makes it somewhat dubious that the disulfide linkages are an essential part of the active group of the gonadotropic hormones. The inactivation produced by large excess of reagent and prolonged reaction time might be ascribed to side reactions.

<sup>3</sup> Gurin, Bachman, and Wilson (9) have recently succeeded in preparing a prolan assaying over 6000 i.u. per mg. Ultracentrifuge studies indicate this preparation to be a homogeneous protein. Analyses of this product gave 1.96 per cent sulfur and 1.92 per cent ash. The authors state the sulfur was probably largely inorganic.

<sup>4</sup> Since the insulin was not crystalline, contaminating proteins may have contributed to the test.

## SUMMARY

1. The pituitary and urinary (prolan) gonadotropic hormones are relatively stable to cysteine and cyanide under experimental conditions which produce inactivation of insulin, and lead to disulfide reduction.

2. Prolonged action (24 hours) and a large excess (10-fold by weight) of cyanide produce partial inactivation of both hormones, and within the error of assay, to the same degree.

3. Prolonged action (48 hours) and a large excess (10-fold by weight) of cysteine hydrochloride produce more than 90 per cent inactivation of the pituitary preparation under experimental conditions which produce 65 to 85 per cent inactivation of prolan.

4. These results are not in accordance with the view that the disulfide linkage is a component of the active group in the pituitary hormone but not in the urinary hormone.

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## RACEMIZATION OF GLUTAMIC ACID WITH HEAT

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Recently we have been confronted with the problem of preparing *dl*-glutamic acid in large quantities. Several methods of racemizing *l*(+)-glutamic acid have been described (1-5), but none of them appears to be suitable for large scale production in the ordinary chemical laboratory. Abderhalden and Kautzsch (6) have investigated the conversion of glutamic acid to pyrrolidonecarboxylic acid by means of heat. They found that *dl*-pyrrolidonecarboxylic acid could be prepared by heating *l*(+)-glutamic acid for some time at 180-200° *dl*-Glutamic acid hydrochloride was formed when the inactive pyrrolidonecarboxylic acid was allowed to stand in hydrochloric acid solution at 37° for several days. Yields were not reported. We have found that small samples (20 gm.) of *l*(+)-glutamic acid can be converted to *dl*-pyrrolidonecarboxylic acid by heating the glutamic acid at 190-195° for 3 hours. In general, larger samples require longer periods of heating for complete racemization. For example, when 500 gm. of glutamic acid were heated in a liter beaker, 5 to 6 hours were necessary. Subsequent hydrolysis of the *dl*-pyrrolidonecarboxylic acid gives *dl*-glutamic acid in good yield (70 per cent).

### EXPERIMENTAL

*Preparation of dl-Glutamic Acid Hydrochloride*—200 gm. of *l*(+)-glutamic acid were placed in an oven at 190-195°. After melting had taken place, heating was continued for 3.5 to 4 hours. Before the brown mass had solidified, it was dissolved in 300 cc. of hot 20 per cent hydrochloric acid, and the hot solution was



filtered through glass. The filtrate was boiled under a reflux for 4 hours, after which it was allowed to stand at room temperature until crystallization had begun. 200 cc. of concentrated hydrochloric acid were now added. The mixture was placed in a refrigerator at  $-10^{\circ}$  overnight.<sup>1</sup> The next day, the precipitated glutamic acid hydrochloride was filtered off. After it was washed with absolute ethyl alcohol and ether, the ether was allowed to evaporate and the material was dried in a desiccator over calcium chloride. Yield, 208.7 gm. (83 per cent); m.p.,<sup>2</sup>  $198^{\circ}$ . (Abderhalden and Kautzsch (6) give  $200^{\circ}$  as the melting point of *dl*-glutamic acid hydrochloride.) A solution made by dissolving 0.5013 gm. in 15 cc. of 9 per cent hydrochloric acid was optically inactive (2 dm. polarimeter tube).

If pure *dl*-glutamic acid hydrochloride is desired, it can be prepared by recrystallization of the crude material from 20 per cent hydrochloric acid.

*Preparation of dl-Glutamic Acid*—208 gm. of the crude *dl*-glutamic acid hydrochloride described above were dissolved in 250 cc. of hot water, and the solution was decolorized with charcoal. After cooling, 455 cc. of approximately 2.5 *N* sodium hydroxide were added. The solution now gave a greenish yellow color with brom-cresol green. After the mixture had remained overnight in the ice box, the crystalline glutamic acid was filtered off and dried. The mother liquor, which now gave a blue color with brom-cresol green, was readjusted to the proper acidity with 10 cc. of concentrated hydrochloric acid, and a second small crop of crystals was obtained. The total glutamic acid isolated was 152.4 gm. (92 per cent; over-all yield, 76 per cent). This material was purified by recrystallization from 900 cc. of water. Yield, 140 gm. (over-all yield, 70 per cent); m.p.,  $198^{\circ}$ . (Fischer, Kropp, and Stahlschmidt (1) give  $199^{\circ}$  (corrected) as the melting point of *dl*-glutamic acid.) A solution prepared by dissolving 0.5510 gm. in 15 cc. of 9 per cent hydrochloric acid was optically inactive when it was read in a 2 dm. polarimeter tube.

<sup>1</sup> The yield will be nearly as high if crystallization takes place at  $0^{\circ}$

<sup>2</sup> The melting points reported in this paper were determined with the Fisher-Johns apparatus (Fisher Scientific Company, Pittsburgh) and are uncorrected.

## SUMMARY

A convenient procedure for the racemization of *l*(+)-glutamic acid is described. It involves the formation and racemization of pyrrolidonecarboxylic acid with heat, with subsequent hydrolysis of the *dl*-pyrrolidonecarboxylic acid to give *dl*-glutamic acid.

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## STUDIES IN PROTEIN METABOLISM

### XIII. THE METABOLISM AND INVERSION OF *d*(+)-LEUCINE STUDIED WITH TWO ISOTOPES\*

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In a recent publication (1) it has been shown, with the aid of deuterium and  $N^{15}$ , that during the biological conversion of *d*(-)-phenylaminobutyric acid into the acetyl derivative of its *l*(+) form most of the nitrogen originally attached to the amino acid was removed and replaced by nitrogen from other sources. Phenylaminobutyric acid is not a physiological compound and is not found in proteins. It is acetylated and excreted in the urine. In work on the "inversion" of this compound one follows simultaneously the process of acetylation. Both processes affect the  $\alpha$ -amino group of the amino acid.

The ability of animals to invert physiological amino acids of "unnatural" configuration has been definitely established (see discussion in (1)). Most experiments have been carried out with indispensable amino acids. Conrad and Berg (2) have followed the inversion of histidine directly by replacing in the diet of growing animals the "natural" histidine by its unnatural isomeride. From the proteins of the animals only natural histidine could be isolated, a finding demonstrating the inversion of the unnatural histidine. Almost all other experiments on inversion of indispensable acids (methionine (3, 4), phenylalanine (4), tryptophane (5), etc.) were based on growth curves. All these studies were thus carried out with animals that were in special need of the natural form of the amino acid and demonstrated that immature rats are able to derive the missing amino acids by inversion.

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The isotope method makes it possible to investigate such processes in adult full grown animals on their ordinary stock diet: If the unnatural form of the amino acid, containing isotope, is added to the diet, and its natural isomeride is isolated from the protein, the presence of isotope in the latter is proof of inversion.

We have recently described experiments with *l*(-)-leucine (6), which was obtained by resolution of a synthetic racemic product containing two independent isotope markers, deuterium attached to the carbon chain and  $N^{15}$  in the amino group. In the present study the metabolic fate of *d*(+)-leucine obtained from the same racemic preparation was followed under exactly the same conditions: weight of animals, basic diet, amount of leucine added, and duration of experiment. To facilitate comparison in Tables I to V we include the values obtained with *l*(-)-leucine. The procedures for the isolation of amino acids were also practically the same, but we have not attempted to isolate as many pure amino acid samples as in the experiment with natural leucine.

In recent work on intermediary metabolism, compounds have been given with isotope contents ranging from 1.2 to 6.7 atom per cent  $N^{15}$ . The isotope concentrations observed in compounds isolated from the animals are a function of those in the material administered. For comparison it is preferable to refer them to a uniform basis. The values in the present paper are accordingly calculated as for 100 per cent  $N^{15}$  in the nitrogen, and 100 per cent deuterium in the hydrogen of the compounds fed. This form of presentation has the advantage that it shows directly what part of the biological reaction product is derived from the administered material. If after feeding leucine with "100" atom per cent  $N^{15}$ , glutamic acid isolated from the proteins contains "2.0" atom per cent  $N^{15}$ , this means that a minimum of 2 per cent of the glutamic acid nitrogen is derived from the leucine administered. The same reasoning applies to work with deuterium. If *d*(+)-leucine with "100" atom per cent deuterium is given, and *l*(-)-leucine isolated from the protein contains "13" atom per cent deuterium, this means that at least 13 per cent of the natural leucine in the protein is derived from the unnatural enantiomorph.

*Balance of Nitrogen of d(+)-Leucine*—The balance of the nitrogen of both leucine isomers is given in Table I. The absorption of the unnatural compound from the intestinal tract was as

good as that of the natural one. However, much less of the isotopic nitrogen was retained, and twice as much was excreted with the urine (58 per cent compared with 27 per cent). The amount of nitrogen introduced into the proteins, though considerable, was correspondingly less. The nitrogen of the unnatural isomer is thus not as well utilized as that of the natural one.

*"Relative Activity" of Organ Proteins (Table II)*—The proteins of all organs have "accepted" nitrogen from the unnatural leucine. As was to be expected from the results of the balance (Table I), the concentration of isotope in the proteins of the organs was in almost every case lower than after feeding of the natural material.

TABLE I

*Balance of Nitrogen Isotope after Feeding Isotopic Leucine*

The values were calculated from the total nitrogen of the fractions and their isotopic concentration.

		Administered N <sup>15</sup> found in different fractions after feeding	
		d(+)-Leucine	l(-)-Leucine
		per cent	per cent
Excreta	Feces	1.7	2.1
	Urine	58.3	27.6
Animal body	Non-protein N	11.7	7.8
	Protein	34.4	57.5
Total isotope recovered . . . . .		106	95

However, the relative activity was about the same for all organs. Isotope concentration was highest in the plasma, that in the viscera intermediate, and that in erythrocytes, muscle, and skin was low.

*Inversion of d(+)-Leucine*—The proteins (after extraction with trichloroacetic acid) were hydrolyzed, and leucine was isolated from the proteins of the liver and of the rest of the body.

The isotope analyses of the leucine preparations are included in Table III. The deuterium content (last line, Table III) in the leucine indicates that the carbon chain has been utilized; *i.e.*, the leucine was inverted and a considerable amount introduced into the proteins. The amount of leucine carbon chain introduced into the liver after the unnatural form is fed is only about 20 per

cent lower than that after the natural isomer. At least 9.8 per cent of the carbon chain of the leucine in the liver proteins was derived from the unnatural leucine of the diet.<sup>1</sup>

On the other hand, the  $N^{15}$  concentration in the leucine from the proteins was very low when compared with the results of the feeding of *l*(-)-leucine. Though a considerable amount of the carbon chain was utilized, most of the nitrogen must have been

TABLE II

*N<sup>15</sup> Concentration in Protein Nitrogen of Blood and Organs from Rats Given Isotopic Leucine*

The values are calculated for a concentration of 100 atom per cent  $N^{15}$  in the administered leucine.

Fraction	$N^{15}$ concentration after feeding	
	<i>d</i> (+)-Leucine	<i>l</i> (-)-Leucine
	atom per cent $N^{15}$ excess	atom per cent $N^{15}$ excess
Blood plasma.....	1.15	1.65
Erythrocytes.....	0.28	0.29
Liver.....	0.89	0.93
Intestinal wall.....	0.76	1.48
Kidney.....	0.97	1.36
Heart.....		0.89
Spleen.....	0.62	1.10
Testes.....		0.76
Skin.....	0.13	0.18
Muscle.....	0.25	0.33
Carcass.....	0.24	0.46

The error of the analysis of  $N^{15}$  is  $\pm 0.003$  atom per cent  $N^{15}$  when concentrations of normal abundance are measured. The error in the calculated values is about 20 times higher; i.e.,  $\pm 0.06$  atom per cent excess.

replaced by nitrogen from other sources. The finding of replacement of original nitrogen during inversion is similar to that observed with phenylaminobutyric acid (1). The probable mechanism responsible for the replacement is discussed later.

*Activity of Amino Groups in Amino Acids of Proteins*—In Table III are also given the  $N^{15}$  concentrations of a number of

<sup>1</sup> The casein of the diet contained an equivalent amount of "natural" leucine. According to our earlier experiments a considerable amount of this material must also have been deposited.

amino acids isolated from the proteins of the liver and the rest of the body. Almost all amino acids investigated contained nitrogen isotope, but less than after the feeding of *l*(-)-leucine. Arginine is an exception. Its higher isotope content after the feeding of the unnatural form of leucine may be associated with the more extensive production of isotopic urea. It was shown in previous papers (see discussion in (6)) that the amidine  $\left( \begin{array}{c} \text{NH}_2\text{C}=\text{NH} \\ | \end{array} \right)$

TABLE III

*Isotope Concentration in Protein Constituents from Rats Given Isotopic Leucine*

The values are calculated for an isotope concentration ( $\text{N}^{15}$  or D) of 100 atom per cent in the administered leucine.

	In liver protein after feeding		In carcass protein after feeding	
	<i>d</i> (+)-Leucine	<i>l</i> (-)-Leucine	<i>d</i> (+)-Leucine	<i>l</i> (-)-Leucine
	atom per cent $\text{N}^{15}$ excess	atom per cent $\text{N}^{15}$ excess	atom per cent $\text{N}^{15}$ excess	atom per cent $\text{N}^{15}$ excess
Amide nitrogen.....	2.45	0.78	0.77	
Glycine.....	0.55	0.73	0.14	
Tyrosine.....		0.50	0.11	0.20
Aspartic acid.....	1.03	1.16	0.24	0.70
Glutamic ".....	1.33	1.85	0.17	0.89
Arginine.....	1.19	0.89		0.24
Leucine.....	0.77	7.92	0.25	1.90
	atom per cent D	atom per cent D	atom per cent D	atom per cent D
Leucine.....	9.77	12.22	1.52	3.33

The error is discussed in Table II.

group of arginine, which according to Krebs and Henseleit (7) is an intermediate in urea formation, always contains considerable concentrations of isotope when isotopic amino acids are administered. It is of interest that in this experiment, as in all our others, glutamic acid has a high isotope concentration. The results definitely prove that the unnatural leucine has yielded nitrogen to other amino acids.

It has been shown that natural leucine, like many other amino acids, is involved in a process of continuous deamination  $\rightleftharpoons$



amination. The present result thus differs only quantitatively from that obtained after feeding of natural leucine.

The  $N^{15}$  concentration in the leucine of the liver is lower than that in the glutamic acid, aspartic acid, and arginine. As leucine is involved in the process of amino shift, it takes up nitrogen from any source that yields nitrogen; *i.e.*, from almost all amino acids. It has been shown that not only the nitrogen of administered natural amino acids, but even that given in the form of ammonia (8) is introduced to some extent into the amino acids of proteins. Some of the nitrogen originally attached to *d*(+)-leucine after its removal must thus have found its way back to leucine in the course of continual deamination  $\rightleftharpoons$  amination. The presence of small

TABLE IV

$N^{15}$  Concentration in Urinary Ammonia and Urea from Rats Given Isotopic Leucine

The values are calculated for a concentration of 100 atom per cent  $N^{15}$  in the administered leucine.

	$N^{15}$ concentration after feeding	
	<i>d</i> (+)-Leucine	<i>l</i> (-)-Leucine
	atom per cent $N^{15}$ excess	atom per cent $N^{15}$ excess
Urea . . . . .	4.19	2.43
Ammonia . . . . .	15.76	3.90

The error is discussed in Table II.

amounts of  $N^{15}$  in the leucine of the proteins thus cannot be taken as indication that any of the original nitrogen had stayed attached to the carbon chain during the process of inversion. The values in Table III taken together make it appear highly probable that all of the nitrogen originally attached to leucine became detached from the carbon chain during the process of inversion; *i.e.*, the *d*(+)-leucine had been completely deaminated. In the second stage of the reaction, amination, a small amount of isotopic nitrogen became reincorporated to form *l*(-)-leucine.

*Amide Nitrogen*—The concentration of isotope in the amide nitrogen from the proteins of liver and body was not only higher than that found after the natural leucine was fed, but also considerably above that of the other amino acids. According to

Leuthardt (9) and Bach (10), the amide group of glutamine as well as the amidine group of arginine is involved in urea formation. The present finding of exceptionally high isotope concentration in both groups may be related to the diminished utilization of the isotopic amino acid nitrogen and its increased excretion as urea (Table IV).

According to Rose (11) leucine, which is an indispensable amino acid, can be replaced in the diet of growing rats by the corresponding  $\alpha$ -hydroxy and  $\alpha$ -keto acids. However, the unnatural isomer, *d*(+)-leucine is reported not to be a substitute for the natural form. The present results show that, while *d*(+)-leucine is converted into the natural isomer in adult animals on an ordinary diet, it is not quite as "effective" as its natural analogue in regard to deposition and nitrogen shift. The differences between the results of Rose and ours may be due to a difference in the age of the animals, or to the mode of experimentation.

#### EXPERIMENTAL

*Preparation of d*(+)-Leucine ( $N^{15}D$ )—The resolution and isolation of pure *l*(-)-leucine from the racemic isotopic mixture have been described in detail (6). The *d*(+) isomer was obtained from the alcohol-insoluble brucine salt of formylleucine. In order to remove any contaminating isotopic *l*(-) isomer, the washing out procedure analogous to that used for the purification of the natural isomer was employed. The *d*(+)-leucine (8.83 gm.) finally obtained contained 10.74 per cent N (Kjeldahl), 6.32 atom per cent  $N^{15}$  excess, and 3.48 atom per cent D.  $[\alpha]_D^{24} = -15.3^\circ$  (3.887 per cent in 20 per cent HCl).

The *l*(-) isomer after the washing out procedure was reported to contain 6.54 atom per cent  $N^{15}$  excess and 3.60 atom per cent deuterium.

*Feeding Experiment*—Four male rats of 1278 gm. total weight were kept in separate cages for 8 days on the same stock diet as used previously. For the following period of 3 days, isotopic *d*(+)-leucine was added to the diet. A mixture containing 2.565 gm. of the amino acid and 171 gm. of diet was completely consumed during the experimental period. The animals maintained a constant weight (the weight at the end of the experimental period was 1285 gm.). The excreta were collected quantitatively.

The animals were killed by exsanguination. The procedure of investigation of excreta and tissues was the same as that previously described.

*Excreta*—The combined urines contained 3.35 gm. of total nitrogen with 0.302 atom per cent  $N^{15}$  excess. A sample of ammonia was obtained by aspiration of the alkaline mixture, and urea was obtained by precipitation with xanthidrol.<sup>2</sup> The isotope concentration in the two fractions obtained after administration of natural and unnatural leucine is given in Table IV.

The results indicate that a much larger fraction of the ammonia was derived from the unnatural than from the natural leucine.

TABLE V

*N<sup>15</sup> Concentration in Non-Protein Nitrogen of Organs from Rats Given Isotopic Leucine*

The values are calculated for a concentration of 100 atom per cent  $N^{15}$  in the administered leucine.

	$N^{15}$ concentration after feeding	
	<i>d</i> (+)-Leucine	<i>l</i> (-)-Leucine
	atom per cent $N^{15}$ excess	atom per cent $N^{15}$ excess
Liver.....	1.84	1.24
Intestinal wall.....	2.55	1.82
Kidney.....	1.98	1.34
Skin.....	0.35	0.41
Muscle.....	0.63	0.46
Carcass.....	0.62	0.57

The error of the analyses is discussed in Table II.

This is probably the result of the rapid deamination of the unnatural isomer by *d*-amino oxidase present in the kidney (12).

The feces contained a total of 0.644 gm. of nitrogen with 0.046 atom per cent  $N^{15}$  excess.

*Investigation of Organs*—The organs were exhaustively extracted with 6 per cent trichloroacetic acid, and the insoluble fraction (protein) and the soluble fractions (non-protein nitrogen) were analyzed. The isotope concentrations in the non-protein nitrogen fractions (Table V) in contrast to those of the protein nitrogen

<sup>2</sup> The authors are indebted to Dr. Samuel Graff for isolation of urea samples. A study of the formation of ammonia and urea from isotopic ammonia and amino acids will be published in a forthcoming paper.

(Table II) of liver, intestinal wall, and kidney are somewhat higher than the concentrations observed after the natural leucine was fed (see also Table I).

The protein fractions were each hydrolyzed in 20 per cent HCl for 24 hours. Small aliquots of all fractions were taken for estimation of nitrogen and  $N^{15}$ . The isotope balance given in Table I was obtained from the following values by calculation.

*Liver*, protein 1.132 gm. of N with 0.056 atom per cent  $N^{15}$  excess, non-protein N 0.120 gm. of N with 0.116 atom per cent  $N^{15}$  excess. *Intestinal tract*, protein 0.574 gm. of N with 0.048 atom per cent  $N^{15}$  excess, non-protein N 0.084 gm. of N with 0.161 atom per cent  $N^{15}$  excess. *Kidney*, protein 0.223 gm. of N with 0.061 atom per cent  $N^{15}$  excess, non-protein N 0.029 gm. of N with 0.125 atom per cent  $N^{15}$  excess. *Spleen*, protein 0.117 gm. of N with 0.039 atom per cent  $N^{15}$  excess, non-protein N 0.018 gm. of N with 0.112 atom per cent  $N^{15}$  excess. *Skin*, protein 0.716 gm. of N with 0.008 atom per cent  $N^{15}$  excess, non-protein N 0.121 gm. of N with 0.022 atom per cent  $N^{15}$  excess. *Muscle*, protein 0.779 gm. of N with 0.016 atom per cent  $N^{15}$  excess, non-protein N 0.101 gm. of N with 0.04 atom per cent  $N^{15}$  excess. *Remaining carcass*, protein 29.57 gm. of N with 0.015 atom per cent  $N^{15}$  excess, non-protein N 4.20 gm. of N with 0.039 atom per cent  $N^{15}$  excess. *Blood serum*, total N 0.179 gm. of N with 0.073 atom per cent  $N^{15}$  excess. *Erythrocytes*, total N 0.745 gm. of N with 0.018 atom per cent  $N^{15}$  excess.

The amino acids listed in Table III were isolated according to procedures previously described (6). The purity was determined by Kjeldahl nitrogen estimation.

*Glycine* (toluenesulfonyl derivative) from carcass, m.p.  $147^{\circ}$ , N 6.21 per cent, 0.009 atom per cent  $N^{15}$ ; from liver, m.p.  $147^{\circ}$ , N 6.18 per cent (calculated 6.11 per cent), 0.035 atom per cent  $N^{15}$  excess. *Tyrosine* from carcass, N 7.79 per cent (calculated 7.74 per cent), 0.007 atom per cent  $N^{15}$  excess. *Aspartic acid* (as anhydrous copper salt) from carcass, N 7.01 per cent (calculated 7.18 per cent), 0.015 atom per cent  $N^{15}$  excess; from liver, 0.065 atom per cent  $N^{15}$  excess. *Glutamic acid* (the hydrochloride) from carcass, N 7.39 per cent (calculated 7.60 per cent), 0.011 atom per cent  $N^{15}$  excess; from liver, 0.084 atom per cent  $N^{15}$  excess.

*Arginine* from the liver was isolated as the flavianate and recrystallized from water. It was decomposed for  $N^{15}$  analysis by extraction of an acidified solution with butyl alcohol. It contained 0.075 atom per cent  $N^{15}$ .

*Leucine* was isolated from the combined livers and from the remaining carcasses via the insoluble copper salts. 55 mg. were

obtained from the liver; N 10.42 (calculated 10.68 per cent). It contained  $0.34 \pm 0.08$  atom per cent deuterium and  $0.049 \pm 0.004$  atom per cent  $N^{15}$  excess. 1.75 gm. were obtained from the remaining carcass;  $[\alpha]_D = +17.1^\circ$ . It contained 53.12 per cent C, 9.51 per cent H, 10.78 per cent N, and 1.98 per cent S. The data indicate a contamination with 9.2 per cent of methionine. The material contained 0.053 atom per cent deuterium and 0.016 atom per cent  $N^{15}$  excess.

Amide nitrogen from the carcass contained 0.049 atom per cent  $N^{15}$  excess; from liver, 0.155 atom per cent  $N^{15}$  excess.

#### SUMMARY

1. Small amounts of leucine of unnatural steric configuration, *d*(+)-leucine, were added to the stock diet of four adult rats for a period of 3 days. The amino acid contained deuterium as a marker of the carbon chain and  $N^{15}$  in the amino group. The experimental conditions were otherwise the same as those, reported previously, when natural leucine was given.

2. The material was well absorbed. Considerably more of the marked nitrogen was excreted in the urine than was observed in the corresponding experiment with *l*(-)-leucine. The amount of isotopic nitrogen introduced into tissue proteins was correspondingly less (34 per cent instead of 57 per cent). All organs had taken part in the "acceptance" of isotopic nitrogen.

3. Amino acids (glycine, tyrosine, aspartic acid, glutamic acid, and arginine) isolated from the proteins of the animals contained isotopic nitrogen, indicating that unnatural leucine, like its natural isomer, yields nitrogen for the process of amino shift among amino acids.

4. Leucine isolated from the proteins of liver and the rest of the body had a considerable content of deuterium, indicating that the carbon chain of the unnatural leucine was used for the formation of natural leucine. The result is proof of the inversion of *d*(+)-leucine in normal adult animals.

5. In contrast to the high deuterium content, the leucine preparations contained only very low concentrations of  $N^{15}$ , lower than in many other amino acids from the same proteins. The result is explained by assuming that inversion took place in two stages: the compound was first *completely* deaminized; in the second stage

(asymmetrical amination) nitrogen from other amino acids was used. In this second part of the reaction a small amount of nitrogen, liberated from the isotopic amino acid, was reintroduced.

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## STUDIES IN PROTEIN METABOLISM

### XIV. THE CHEMICAL INTERACTION OF DIETARY GLYCINE AND BODY PROTEINS IN RATS\*

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It has been found with the aid of isotopes that the nitrogen of some dietary amino acids (*dl*-tyrosine (1), *l*(-)-leucine (2), *d*(+)-leucine (3), and even ammonia (4, 5)) enters into various chemical reactions which proceed normally with the body proteins. In the course of these processes a large fraction of the dietary nitrogen becomes fixed in the proteins. The nitrogen excreted by animals in nitrogen equilibrium is not merely that of the diet but a sample of the metabolic pool originating from interaction of dietary nitrogen with the relatively large quantities of reactive protein nitrogen.

When isotopic *dl*-tyrosine or isotopic *l*(-)-leucine was given as a minor component of the diet to non-growing animals in nitrogen equilibrium, the corresponding amino acids (tyrosine, leucine, etc.) isolated from the body protein had a very high isotope concentration, indicating a replacement of amino acids in the proteins by the same type of free amino acids. Amino acids other than those administered, *i.e.* glycine, glutamic acid, aspartic acid, etc., had a lower but appreciable N<sup>15</sup> concentration. Nitrogen originally attached to the carbon skeleton of leucine or tyrosine must have been removed and attached to other chains to form new amino acids. These in turn must have replaced the same type of amino acid in the proteins. Glutamic and aspartic acids always had a higher isotope concentration than other amino

\* This work was carried out with the aid of grants from the Josiah Macy, Jr., Foundation and the Rockefeller Foundation.



acids, indicating a high metabolic activity of these protein constituents. The continuous process of deamination and amination at the  $\alpha$ -carbon atom was observed to occur not only with dispensable amino acids but also with some indispensable ones such as leucine and histidine (6). The amino acid lysine seems to play an exceptional rôle; in contrast to the other protein constituents thus far investigated, lysine is never found to contain nitrogen from other amino acids.

Besides the  $\alpha$ -amino group, two other nitrogenous groupings of the proteins were found to be involved in continuous reactions, the amide group and the amidine group of arginine. As free arginine (7) and glutamine (8, 9) have been shown *in vitro* to play a rôle in urea formation, the presence of isotope in the amide and amidine groups of the proteins was taken as indication that these parts of the protein molecule play an analogous rôle *in vivo*, probably in connection with continuous liberation and reintroduction of the amino acids to which they are attached.

The process of transfer of nitrogen from one amino acid to others is very rapid. The amount of isotopic nitrogen incorporated in the protein by this transfer process is much higher than that which enters the protein in conjunction with the carbon chain of the same amino acid to which the isotopic nitrogen was originally attached. When *l*(-)-leucine was administered, only one-third the isotopic nitrogen in the total protein was found attached to the leucine carbon chain; the remaining two-thirds was present in other protein constituents. Similar results were obtained after *dl*-tyrosine was fed (1). All the reactions indicated with isotopes must have been so balanced as to avoid changes, for the total amounts and structure of the various proteins remain constant within narrow limits in non-growing, normal animals.

In the present study, the fate of glycine in normal rats has been investigated under conditions analogous to those of the previous experiments. Four full grown rats were given our customary stock diet, the protein of which is casein. This protein contains not more than traces of glycine (10). For a period of 3 days a moderate amount of isotopic glycine corresponding to 23 mg. of N per rat per day was added to the diet. After the animals had consumed the mixture, they were killed by exsanguini-

nation and the bodies worked up in a manner similar to that of the previous experiments.

The amount of nitrogen (23 mg. per rat per day) added as glycine to the diet is the same as that added to the same basic diet in previous experiments (tyrosine (1), *l*(-)-leucine (2), *d*(+)-leucine (3), ammonium citrate (5)). The results of all these experiments are thus directly comparable when calculated as outlined in Paper XIII (3) for 100 atom per cent  $N^{15}$  in the compound (in this case glycine) added to the diet.

TABLE I

*Balance of Nitrogen Isotope after Feeding Isotopic Glycine*

The values were calculated from the total nitrogen of the fractions and their isotopic concentration. The animals consumed 879 micro-equivalents\* of  $N^{15}$  excess.

		$N^{15}$	Fraction of $N^{15}$ administered
		micro- equivalents*	per cent
Excreta	Feces	22	2.5
	Urine	357	40.6
Animal body	Non-protein N	102	11.6
	Protein N	390	44.3
Total isotope recovered. ....		871	99.0

\* 1 microequivalent of  $N^{15}$  = 15  $\gamma$  of  $N^{15}$ .

The fate of dietary glycine nitrogen was found to be generally the same as that of the other compounds investigated. Some processes, namely excretion and the introduction of nitrogen into amide and amidine groups, seem to be somewhat faster, while the transfer to  $\alpha$ -amino nitrogen may be somewhat slower. These quantitative differences will be discussed later.

*Balance of Glycine Nitrogen*—The excreta were quantitatively collected and investigated as described before. The tissues of the animals were exhaustively extracted with 6 per cent trichloroacetic acid. The total amount of nitrogen and the isotope content of each fraction were determined. In Table I is given the distribution of isotopic nitrogen, *i.e.* of ingested glycine nitrogen,

among the different fractions. The material was well absorbed<sup>1</sup> but only 41 per cent of the administered marked nitrogen appeared in the urine. Most of the remainder (44 per cent) had replaced nitrogen in the proteins and a small amount (11 per cent) was recovered in the non-protein nitrogen fraction. The total recovery of isotope was good.<sup>2</sup> The present findings taken together with the similar results obtained when other isotopic compounds were fed convince us that *we are dealing with a reaction typical for most dietary amino acids.*

TABLE II

*N<sup>15</sup> Concentration in Protein Nitrogen of Blood and Organs from Rats Given Isotopic Glycine*

Organ	Total N	Amount of N <sup>15</sup>	N <sup>15</sup> concentration*
	milliequivalents	microequivalents	atom per cent N <sup>15</sup> excess
Erythrocytes.....	61.2	12.2	0.44
Serum.....	16.1	12.9	1.77
Liver.....	79.9	50.3	1.40
Intestinal tract.....	32.0	14.1	0.97
Brain.....	7.4	1.3	0.38
Carcass†.....	2302.0	299.3	0.29
Total.....	2498.6	390.1	

The error of the analysis of N<sup>15</sup> is  $\pm 0.003$  atom per cent N<sup>15</sup> when concentrations of normal abundance are measured. The error in the calculated value is about 20 times higher; *i.e.*,  $\pm 0.06$  atom per cent excess.

\* The values in this column are calculated for an N<sup>15</sup> concentration of 100 atom per cent in the glycine added to the diet.

† This fraction represents all the remainder of the body.

*Relative Activity of Organ Proteins*—In Table II are given the isotope concentrations in the nitrogen of different organs. It was pointed out before (2) that these values indicate the relative

<sup>1</sup> It is highly questionable whether the small amount of isotope in the feces found in all such experiments represents unabsorbed food material. It is more probable that the isotopic nitrogen had entered the intestinal lumen with the proteins of the intestinal secretæ, which, like the proteins and the other nitrogenous constituents of the animals, must have contained N<sup>15</sup>.

<sup>2</sup> The precision of recovery (99 per cent) of the isotope is fortuitous. We estimate the error of such balances to be  $\pm 10$  per cent.

chemical activity of the proteins in regard to the acceptance of glycine nitrogen. The results with glycine are thus similar to those with leucine (2, 3); the proteins of serum, liver, and intestinal tract have a high "activity," while that of the erythrocytes (hemoglobin) and of the muscle and skin (carcass) is low. The latter organs, however, played the most important rôle in the total replacement process, owing to their large size and their high total nitrogen content.

*Replacement of Glycine in Proteins by Dietary Glycine*—The proteins of liver, intestinal tract, and the combined proteins of

TABLE III

*N<sup>15</sup> Concentration in Protein Constituents from Rats Given Isotopic Glycine*

The values are calculated for an N<sup>15</sup> concentration of 100 atom per cent in the glycine added to the diet.

	Liver	Intestinal tract	Carcass
	atom per cent N <sup>15</sup> excess	atom per cent N <sup>15</sup> excess	atom per cent N <sup>15</sup> excess
Glycine.....	8.86	4.22	1.04
Tyrosine.....	0.47		0.13
Arginine.....	0.78		0.09
Ornithine from arginine.....	0.24		
Ammonia " ".....	1.53		
Glutamic acid.....	0.89		0.27
Aspartic ".....	0.73		0.20
Amide N.....	1.44		0.56

The error is discussed in Table II.

muscle and skin were hydrolyzed and samples of several pure amino acids isolated. The N<sup>15</sup> concentrations of these samples are given in Table III. The glycine isolated had an isotope concentration much higher than that of any other protein fraction, indicating the direct introduction of glycine. In the previous experiment with leucine the incorporation into the proteins of the carbon chain was established by the presence of an independent isotope, deuterium. This mode of experimentation is not feasible in the case of glycine, as the hydrogen atoms of its methylene group are "semilabile" (11-13), but the parallelism in the results of

this and the earlier experiments leaves no doubt as to its interpretation.

The values given in Table III indicate that in the 3 day period a minimum of 8.8 per cent of the glycine present in the liver proteins has been replaced by dietary glycine. The corresponding values for intestinal tract and for muscle and skin are 4.2 and 1 per cent respectively. This calculation presupposes that none of the isotopic nitrogen originally attached to the carbon chain had been replaced by normal nitrogen from other sources. The nitrogen of glycine is, however, involved in such transfer processes (see below). Part of the isotope must have been replaced by normal nitrogen in the course of these reactions. The amounts of glycine carbon chain newly introduced into the proteins must thus have been higher than is indicated by the concentration of nitrogen isotope.

While these values indicate how much of the glycine in the protein had been replaced, they do not indicate what fraction of the dietary glycine became incorporated in the proteins. To calculate this value it is necessary to know the total amount of glycine in dietary and animal proteins in addition to their isotope contents. All data except the content of glycine in the total protein of rats are known. We have determined the latter by the "isotope dilution" method (14), in a manner analogous to the determination of leucine in rat protein (2). The nitrogen of the total protein of rats was found to contain 10.1 per cent of glycine nitrogen. On the basis of these data it was calculated that a minimum of 13 per cent of the dietary glycine was directly introduced in the proteins as glycine. This value is also based on the assumption that no isotope in the glycine has been replaced by normal nitrogen. The actual amounts introduced must have been higher.

Glycine is a dispensable amino acid and can be synthesized by rats (15). It is probable that newly synthesized glycine in addition to that of the diet also takes part in the replacement process.

*Activity of  $\alpha$ -Amino Group*—The nitrogen of tyrosine, glutamic acid, and aspartic acid (Table III) contained considerable concentrations of isotopic nitrogen. In agreement with all earlier experiments, the concentration of  $N^{15}$  in glutamic acid is highest. These findings indicate that these amino acids had accepted nitrogen originally present in glycine. This is additional evidence for

the occurrence of continuous nitrogen shifts in the  $\alpha$ -amino groups of the amino acids of proteins. As stated in previous papers (1, 3), this type of experiment does not disclose the mechanism responsible for the nitrogen transfer (synthesis and degradation of amino acids, transamination, reductive amination of keto acids in the presence of ammonia, etc.).

Ornithine isolated from the arginine contained a small but significant concentration of isotope. The marker may have been located in the  $\alpha$ -,  $\delta$ -, or both amino groups. Ammonia liberated from arginine by treatment with alkali contained a rather high concentration of isotope. This nitrogen must have been present

in the  $\text{NH}_2\text{—}\overset{\textstyle |}{\text{C}}\text{=NH}$  (amidine) group of arginine. The amide nitrogen of the proteins had about the same isotope concentration. These high concentrations in both fractions may be correlated with the formation and excretion in the urine of somewhat larger amounts of isotope in the form of urea than was found after addition of other natural amino acids. The rôle of protein arginine in urea formation has been discussed extensively before (1, 2).

*Glycine As Nitrogen Donor and Acceptor*—The results of the present experiment show that glycine yields nitrogen to the  $\alpha$ -amino group of other amino acids. In earlier experiments when isotopic  $l(-)$ - or  $d(+)$ -leucine (2, 3) or ammonia (4, 5) was given, the glycine isolated from the proteins contained marked nitrogen. Glycine thus belongs to the group of amino acids which yield as well as accept nitrogen from other compounds. As mentioned before, the mode of transfer is not yet established. In the case of a dispensable amino acid like glycine, it is possible that the "acceptance" of nitrogen from other compounds occurs in the course of the total synthesis of the amino acid.

The rôle of glycine as a precursor of body creatine has already been discussed in a note (16).

#### EXPERIMENTAL

*Preparation of Glycine ( $\text{N}^{15}$ )*—The isotopic glycine employed in this experiment was prepared via isotopic ethyl phthalimido acetate by the method previously described (17). The substance contained 4.50 atom per cent  $\text{N}^{15}$  excess.

*Feeding Experiment*—Four male rats kept separately in metab-

olism cages were maintained on our stock diet containing casein (1, 2, 5). The animals had a combined weight of 1262 gm. After 6 days isotopic glycine was added to the diet daily for 3 days. During the experimental period, 176 gm. of diet containing 1.466 gm. of isotopic glycine, or 0.273 gm. of glycine nitrogen, had been consumed. The animals maintained a constant weight (their weight at the end of the experimental period was 1286 gm.). The excreta were collected quantitatively. The animals were killed by exsanguination. The procedure of investigation of excreta and tissues was the same as that described before.

*Excreta*—The combined urines contained 3.91 gm. of total nitrogen with 0.128 atom per cent  $N^{15}$  excess. A sample of ammonia was obtained by aspiration of the alkaline mixture, and urea was obtained by precipitation with xanthidrol.<sup>3</sup> These samples contained 0.148 and 0.123 atom per cent  $N^{15}$  excess respectively. The feces were combined with the contents of the intestinal tract. The material contained 0.551 gm. of total nitrogen with 0.057 atom per cent  $N^{15}$  excess.

*Investigation of Organs*—The organs were exhaustively extracted with 6 per cent trichloroacetic acid, and the insoluble fractions (protein) as well as the soluble fractions (non-protein nitrogen) were analyzed. The liver non-protein nitrogen contained 0.106 gm. of total nitrogen with 0.096 atom per cent  $N^{15}$  excess. The intestinal tract non-protein nitrogen contained 0.088 gm. of total nitrogen with 0.067 atom per cent  $N^{15}$  excess. The carcass non-protein nitrogen contained 4.21 gm. of total nitrogen with 0.030 atom per cent  $N^{15}$  excess (see Table I).

Each of the protein fractions was hydrolyzed in 20 per cent hydrochloric acid for 24 hours. Small aliquots of all fractions were taken for estimation of total nitrogen and  $N^{15}$ . The  $N^{15}$  concentrations found in the various organs in atom per cent  $N^{15}$  excess are as follows: erythrocytes 0.020, serum 0.080, liver 0.063, intestinal tract 0.044, brain 0.017, carcass 0.013 (compare Table II).

The isotope balance given in Table I was calculated from the nitrogen contents of each fraction and their respective  $N^{15}$  concentrations. For the total nitrogen contents of the organs see Table II.

<sup>3</sup> The authors are indebted to Dr. Samuel Graff for isolation of the urea samples.

The amino acids listed in Table III were isolated according to procedures previously described (2). The purity was determined by Kjeldahl nitrogen estimation.

*Glycine* (toluenesulfonyl derivative) from carcass had a melting point of 147–148°, N 5.95 per cent, 0.047 atom per cent  $N^{15}$  excess; from liver, m.p. 147–148°, N 6.19 per cent, 0.399 atom per cent  $N^{15}$  excess; from intestinal tract, m.p. 147–148°, N 6.21 per cent, 0.190 atom per cent  $N^{15}$  excess. *Tyrosine* from carcass gave N 7.62 per cent, 0.006 atom per cent  $N^{15}$  excess; from liver, N 7.88 per cent, 0.021 atom per cent  $N^{15}$  excess.

*Arginine* was isolated as the flavianate and recrystallized from water. For  $N^{15}$  analysis the flavianate was decomposed by extraction of an acidified solution with butyl alcohol. From carcass 0.004 atom per cent  $N^{15}$  excess was obtained; from liver 0.035 atom per cent  $N^{15}$  excess. 830 mg. of arginine flavianate isolated from the liver protein were degraded with an excess of barium hydroxide as previously described (2). A sample of the ammonia collected during the reaction contained 0.069 atom per cent  $N^{15}$  excess. *Ornithine* was isolated from the reaction mixture as the monohydrochloride, which was then converted to the dibenzoyl derivative. Yield 147 mg., m.p. 188–188.5°, N 8.33 per cent, 0.011 atom per cent  $N^{15}$  excess.

*Glutamic acid* (as the hydrochloride) from carcass gave N 7.61 per cent, 0.012 atom per cent  $N^{15}$  excess; from liver, N 7.88 per cent, 0.040 atom per cent  $N^{15}$  excess.

*Aspartic acid* (as the anhydrous copper salt) from carcass gave N 7.00 per cent, 0.009 atom per cent  $N^{15}$  excess; from liver, N 7.08 per cent, 0.033 atom per cent  $N^{15}$  excess.

*Amide nitrogen* from carcass gave 0.025 atom per cent  $N^{15}$  excess; from liver, 0.065 atom per cent  $N^{15}$  excess.

*Determination of Glycine in Protein of Rat*—Glycine was estimated by the "isotope dilution" method (14). The trichloroacetic acid-extracted proteins obtained from one adult normal male rat were hydrolyzed by boiling under a reflux in 800 cc. of 20 per cent hydrochloric acid for 24 hours. The solution was made up to a volume of 2000 cc. and 250 cc. aliquots (A and B) each containing 1018 mg. of nitrogen were taken for analysis. To Aliquot A was added 70.0 mg. of isotopic glycine, and to Aliquot B 71.1 mg. of the same preparation containing 4.56 atom



per cent  $N^{15}$  excess. The solution was then decolorized with norit and concentrated to a syrup *in vacuo*. Glycine was precipitated as the trioxalatochromiate as described by Bergmann and Niemann (18). The precipitate was dissolved in 100 cc. of water and the nitrogen content (6.5 mm) determined by Kjeldahl estimation. The solution was brought to pH 6 by addition of dilute sodium hydroxide, and then treated with 2.5 gm. of toluenesulfonyl chloride (13 mm) dissolved in ether. The mixture was stirred continuously and 13 cc. of 2 N sodium hydroxide were added over a period of 2 hours. Stirring was continued 2 hours longer. The solution was extracted three times with small amounts of ether, filtered, acidified with dilute sulfuric acid, and extracted with ether in a continuous extractor. The crystalline residue obtained after removal of ether was recrystallized twice from hot water and then twice from acetone by precipitation with petroleum ether.

From Aliquot A 0.989 gm. of toluenesulfonyl glycine were obtained, m.p. 147–148°, N 6.12 per cent, 0.518 atom per cent  $N^{15}$  excess; from B, 1.030 gm., m.p. 147–148°, N 6.06 per cent, 0.538 atom per cent  $N^{15}$  excess. The amount of glycine present in Aliquot A may be calculated from the expression  $((4.56/0.518) - 1) \times 70.0$ , and was found to be 546 mg. of glycine, or 101.8 mg. of glycine nitrogen. Analogous calculations from the results obtained in Aliquot B give a value of 10.2 per cent.

*Calculation of Dietary Glycine Deposited in Protein*—As was shown above, 10.1 per cent of the protein nitrogen (34.98 gm.) is glycine nitrogen, which corresponds to 18.9 gm. of glycine in four rats. The  $N^{15}$  content of the glycine isolated from the carcass protein was 0.047 atom per cent  $N^{15}$  excess, which we assume in this calculation to represent the average value for the glycine of the entire animal, since the carcass contained most of the nitrogen (see Table II). The animals consumed 1.466 gm. of isotopic glycine containing 4.50 atom per cent  $N^{15}$  excess. The deposited glycine has, therefore, been “diluted” 96 times ( $4.50/0.047$ ) by admixture with normal nitrogen in the carcass glycine. 197 mg. of the body glycine must, therefore, have been of dietary origin; i.e., 13 per cent of the ingested amino acid has been deposited in 3 days.

*Deposition of  $N^{15}$  in Protein Constituents Other Than Glycine*—44.3 per cent of the isotopic nitrogen administered as glycine was

found in the body protein (Table I). This corresponds to 0.390 milliequivalent of  $N^{15}$ . Since 10.1 per cent of the body protein nitrogen was glycine nitrogen, and there were 2499 milliequivalents of total protein nitrogen in the body (see Table II), 252.4 milliequivalents were total glycine nitrogen. This contained 0.047 atom per cent  $N^{15}$  excess, 0.119 milliequivalent of  $N^{15}$ , which is 31 per cent of the total  $N^{15}$  present in the body protein. The rest, 69 per cent, must therefore have been present in other amino acids in the protein.

#### SUMMARY

1. Four adult non-growing rats were kept on an ordinary stock diet to which was added during the last 3 days an amount of glycine corresponding to 23 mg. of nitrogen per rat per day. The added glycine contained 4.5 atom per cent  $N^{15}$  excess. All experimental conditions were otherwise identical with those employed when the metabolism of other isotopic amino acids was investigated. The excreta and organs of the animals were worked up in the same fashion as in the earlier experiments.

2. The glycine was excellently absorbed, but only 40 per cent of the isotopic nitrogen appeared in the urine. Almost all of the rest had replaced a corresponding amount of body nitrogen. A small amount (11 per cent) was found in the non-protein nitrogen fraction, and the bulk in the proteins. The relative "activity" of the various body proteins in regard to the "acceptance" of glycine nitrogen was about the same as their activity towards  $l(-)$ -leucine nitrogen. Muscle and skin took the greatest share in the uptake of dietary nitrogen.

3. From the hydrolysates of the proteins of liver, intestinal wall, and the remainder of the bodies, several samples of pure amino acids were isolated. The glycine samples had the highest isotope content, indicating that free glycine had replaced glycine in protein linkage. In the course of 3 days a minimum of 8.8 per cent of the glycine in the liver proteins was replaced by dietary glycine. The corresponding values for the proteins of the intestinal wall and of the remainder of the body are 4.2 and 1 per cent respectively.

4. In addition to glycine, other amino acids (glutamic acid, aspartic acid, tyrosine, arginine) of the proteins contained isotope, indicating the transfer of glycine nitrogen to the carbon chain of

other amino acids. Glycine, like many other amino acids, is involved in the continuous process of yielding nitrogen to and accepting nitrogen from other amino acids.

5. The results of the glycine experiment are thus generally the same as those obtained with other amino acids. Only quantitative differences were observed. The theoretical discussion presented in our earlier paper (2) thus holds also for the explanation of the present results.

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# THE DETERMINATION OF MANGANESE IN ORGANIC MATERIAL CONTAINING LARGE AMOUNTS OF CALCIUM AND CHLORIDES

## THE DETERMINATION OF MANGANESE IN THE MOUSE AND IN MILK

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Studies on the metabolism of manganese in the mouse frequently require making an analysis of this element in the ash of the whole animal. The large amounts of calcium and chloride present in this ash present a special problem in the quantitative determination of manganese. The chlorides must be removed, but in doing this one must avoid forming insoluble calcium salts, because manganese is difficult to remove by washing from materials such as calcium sulfate and calcium phosphate.

A modification of the Davidson and Capen (1) method is used in this work. The Davidson and Capen method is suitable for analysis of manganese in plants which contain relatively small amounts of chlorides, but to apply it to ash high in chlorides it was found that special modifications had to be introduced. The present method consists in oxidizing the manganese to permanganic acid with potassium persulfate and then determining the quantity colorimetrically. Complete conversion of manganese to permanganic acid requires the removal of chlorides. This is accomplished by dissolving the ash in concentrated nitric acid, adding sulfuric acid, and evaporating the solution to dryness. By avoiding an excess of sulfuric acid it is possible to prevent the formation of calcium sulfate, thus making filtration unnecessary.

### *Method*

The mice are killed by asphyxiation with illuminating gas, thoroughly washed with distilled water, and the alimentary tracts

removed and discarded. The livers are analyzed separately. The material is dried in an electric oven at  $105^{\circ}$  and ashed in silica beakers, in a silica-lined and thermostatically controlled muffle furnace. The use of silica throughout insures against contamination from utensils or from the walls of the furnace, while the accurately controlled temperature eliminates the danger of overheating. Tests show that a temperature of  $650^{\circ}$  is insufficient to ash organic matter completely but that a temperature greater than  $700^{\circ}$  is unnecessary. A higher temperature might even cause the formation of oxides which would be difficult to dissolve. After about 8 hours of heating at slightly less than  $700^{\circ}$ , the ashing is completed and the material, when cool, is dissolved in 10 cc. of concentrated nitric acid. 4 drops of concentrated sulfuric acid are added and the solution evaporated to dryness over an open flame to remove the chlorides. More sulfuric acid is unnecessary and undesirable, since it will form more calcium sulfate than can be dissolved. The residue is then dissolved in 12 cc. of 25 per cent nitric acid and the solution made to volume. Since the manganese content of a mouse fed on a manganesc-restricted diet is very small, the final volume should not be greater than 20 cc. For mice fed on a normal diet or for those which have had manganese added to the diet the volume is made to 50 cc. The amount of nitric acid used in dissolving the residue should not be greater than 15 per cent of the final volume, because the author (2) has found that this is near the upper limit for developing the maximum intensity of permanganic acid color. To a measured amount of the solution in a graduated centrifuge tube are added 0.2 cc. of 0.25 per cent silver nitrate, as a catalyst, and 0.25 gm. of potassium persulfate. If the ash is thought to be low in manganese and the final volume is made to 20 cc. in order to concentrate the manganese as much as possible, 10 cc. of the undiluted solution should be used. If, however, the animal had been fed on a normal diet or if manganese had been added to the diet, then from 2 to 5 cc., when diluted to 10 cc. with distilled water, will contain enough manganese to give a convenient depth of color for reading. When the tubes are heated in a water bath, the pure permanganic acid color, free from turbidity, appears within a few minutes and will persist for weeks without fading.

The color is compared with a standard similarly treated, in Nessler tubes of 10 cc. capacity, graduated in tenths of a cm., and 12 cm. in length. Most accurate readings can be made in these tubes when not more than 0.02 mg. of manganese in 10 cc. is measured. The oxidation may be brought about in the presence of nitric acid, sulfuric acid, or phosphoric acid. However, the

TABLE I  
*Results of Analyses and Recovery of Added Manganese in  
Dry Tissue of Mouse*

Material	Whole mouse	Liver	Weight of dry sample used	Mn added	Mn found	Mn recovered
	gm.	gm.	gm.	mg.	mg.	per cent
Whole mouse minus liver (0.3 mg. Mn per day for 90 days)	6.5		0.26 0.26 0.26	0.0018	0.0024 0.0027 0.0043	97.2
Liver		0.45	0.45		0.0053	
Whole mouse minus liver (fed on dog biscuits)	5.8		1.16 1.16 1.16	0.0035	0.0018 0.0016 0.0053	102.8
Liver		0.33	0.33		0.0025	
Whole mouse minus liver (fed on dog biscuits)	6.7		1.34 1.34 1.34	0.0028	0.0017 0.0019 0.0045	96.4
Liver		0.42	0.42		0.0032	
Whole mouse minus liver (milk-fed)	7.4		7.40		0.0035	
Liver		0.43	0.43		0.0015	
Whole mouse minus liver (milk-fed)	6.3		6.30		0.0028	
Liver		0.39	0.39		0.0012	

writer has observed that the resulting color has a different tint with each acid. Therefore for accurate readings nitric acid should be used in the standard, since that acid is used in the sample.

Sufficient data are given in Tables I and II to show the applicability of the procedure. Table I shows the results of the analyses of manganese in five mice. Three were fed on dog biscuits, and one of the three had 0.3 mg. of manganese added to

its drinking water each day for 90 days. The other two received only milk supplemented with iron and copper.

Mice kept on a manganese-restricted diet in this laboratory are fed nothing but milk to which iron and copper are added; therefore it was necessary to know the manganese content of this food. Since both the ash of the mouse and that of milk contain very small amounts of manganese and large amounts of calcium and chlorides, the procedure described for the determination of manganese in the mouse can be applied directly to that of milk. The ash from 100 cc. of milk contains enough manganese to give

TABLE II

*Results of Analyses and Recovery of Added Manganese  
in Three Samples of Skim Milk*

Each determination was carried out on 100 cc. of milk.

Skim milk sample No.	Mn added	Mn found	Recovery of added Mn
	mg.	mg.	per cent
I		0.0023	
		0.0026	
	0.0008	0.0032	93.7
II		0.0033	
		0.0038	
	0.0012	0.0048	104.2
III		0.0038	
		0.0035	
	0.0015	0.0051	96.6

a color of sufficient intensity for satisfactory reading if the volume is made to 15 cc.

Table II shows the analyses of three separate samples of milk. The manganese for recovery was added before the material was ashed. In the whole animal this manganese was added to an aliquot part of the solution before the color was developed. The recovery of added manganese was satisfactory in all cases, and there was never any interference from calcium or chlorides. In analyzing tissues containing less calcium or chlorides than is found in the whole animal or in milk no change in the procedure is necessary.

## SUMMARY

A procedure is presented for the determination of small amounts of manganese in ash which is high in calcium and chlorides. An essential feature in the method consists in removing chlorides with a minimum amount of sulfuric acid, thus preventing the formation of calcium sulfate. Filtration, therefore, becomes unnecessary and the danger of losing manganese in the process of filtering and washing is eliminated.

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# THE EFFECTS OF LIVER AND PANCREAS EXTRACTS UPON FAT SYNTHESIS AND METABOLISM

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In previous papers (1-3) we have described the influence of thiamine upon fat synthesis in rats and in pigeons, and the effects of other B vitamins and of choline upon the distribution of the fat thus formed. In a continuation of this work we desired to investigate the effects of pantothenic acid and of factor W. Since a pure preparation of the former was not readily available and since the latter has not yet been isolated, an extract of beef liver which has been shown to contain both these factors (4, 5) was employed. Production of fatty livers in rats fed this extract led to a study of methods for the prevention of such fat deposition in the liver.

## *Methods*

Rats were employed as test animals. The strain, age, and care were the same as previously reported (3). Basal Diet 1 (3) was used throughout. To deplete the rats of their stores of B vitamins and of fat, they were fed only the basal diet for 3 weeks. At the end of this period, body weight and body fat had been definitely diminished. During a subsequent period of 1 week various combinations of supplements were administered to different groups. Thiamine, riboflavin, vitamin B<sub>6</sub>, and choline were given by subcutaneous injection, since previous trials had shown that they were as effective by this route as by mouth and that more accurate dosage of the animals was possible. The liver fraction and supplements other than those previously mentioned were mixed with the food. Vitamin and choline supplements were given in the following amounts per rat per day unless otherwise indicated: thiamine hydrochloride (Merck)

12.5 micrograms, riboflavin (Hoffmann-La Roche) 10.0 micrograms, vitamin B<sub>6</sub> (kindly donated by Merck and Company and by E. R. Squibb and Sons) 20 micrograms, choline hydrochloride (British Drug Houses) calculated as choline base 10 mg., liver fraction 0.5 cc., brewers' yeast powder (Mead Johnson and Company), lipocain (generously furnished by Eli Lilly and Company), and rice polish concentrate (Labco) as indicated.

The liver fraction was prepared as follows: 70 kilos of fresh beef liver were minced and stirred for 1 hour with 135 liters of water containing 70 cc. of sulfuric acid. The mixture was heated to 70° and filtered while hot through a linen bag. The liver residue was reextracted with 112 liters of water. The combined filtrates were concentrated *in vacuo* to one-eighth of the original volume and the concentrate was mixed with sufficient ethyl alcohol to give an alcohol concentration of 52 per cent. A precipitate settled out and was removed by filtration. The filtrate was concentrated *in vacuo* to one-tenth of its volume and the concentrate poured into sufficient absolute ethyl alcohol to give an alcohol concentration of 90 per cent. After removal of a heavy precipitate, the liquid was distilled *in vacuo* until free of alcohol. The final volume was adjusted so that 0.5 cc. contained the material derived from 10 gm. of liver.

The rats were killed by stunning, the livers removed, and total crude fatty acids in the livers and bodies were determined by methods previously published (1, 3), although in some cases livers from a group of animals were combined for analysis and a procedure similar to that reported for the bodies was used. All results are averages for groups of ten rats.

#### EXPERIMENTAL

##### *Effect of Liver Extract upon Fat Synthesis*

*Series I*—The effect of the liver fraction when given as the only supplement and with various combinations of other supplements was investigated. The weight changes during the supplement period and the total crude fatty acids in the liver and body at the end of the experiment are given in Table I. When the liver fraction was given, there was a marked increase in weight, the percentage of liver fat was abnormally high, and the body fat increased.

*Series II*—The liver fraction contained significant amounts of thiamine and choline. We are indebted to Dr. D. G. H. MacDonald and Mr. J. G. Truax of this laboratory for the determination of the amounts. By means of the thiochrome procedure (6) the liver fraction was found to contain 15 micrograms of thiamine per cc. By the method of Fletcher, Best, and Solandt (7) the choline content was 20 mg. per cc. Animals receiving supplements of liver fraction and of thiamine secured a total amount of this vitamin which was approximately double the quantity or-

TABLE I  
*Effect of Liver Fraction upon Fat Synthesis*

Series No.	Supplements	Average individual weight change in supplement wk.	Total crude fatty acids in	
			Liver	Body
		gm.	per cent	per cent
I	None, 3 wks. depletion		4.4	2.1
	Liver fraction	+25	9.0	8.4
	" " + thiamine	+34	16.0	8.9
	" " + " + choline	+35	14.7	8.1
	" " + " + " +	+34	11.4	9.1
	flavin			
II	Same + vitamin B <sub>6</sub>	+36	13.5	5.5
	Thiamine (12.5 γ)	+3	13.7	4.8
	" (12.5 γ) + choline (10 mg.)	+5	3.8	5.0
	" (25 γ)	+5	14.7	4.8
	" (25 γ) + choline (10 mg.)	+6	3.3	5.3
	" (25 γ) + " (20 γ)	+6	3.8	5.5

dinarily employed by us. It was necessary to determine whether the increase in the amount of thiamine was responsible for the effect produced by the liver fraction. The result secured with a dose of thiamine double our customary amount was studied in this series. The effect of choline in preventing the deposition of liver fat caused by thiamine was also investigated, with either the amount of choline ordinarily employed by us or double this quantity. The amount of choline supplied in the liver fraction was sufficient, in itself, to prevent fatty livers caused by thiamine. It is obvious that the fatty livers caused by administration of liver extract are different from those produced by thiamine alone.

*Prevention of Development of Fatty Livers Caused by Liver Extract  
(Table II)*

*Series III*—The administration of customary doses of choline (Series I) did not markedly diminish the deposition of liver fat

TABLE II

*Prevention of Development of Fatty Livers Caused by Liver Extract*

Series No.	Supplements	Total crude fatty acids in	
		Liver	Body
		per cent	per cent
III	None, 3 wks. depletion	2.6	1.9
	Liver fraction + thiamine	15.6	6.8
	" " + " + choline (10 mg.)	13.3	6.7
	" " + " + " (40 " )	9.5	6.8
	" " + " + " (90 " )	12.1	6.8
IV	After 3 wks. depletion period		
	Fox Chow	4.7	4.2
	" " + liver fraction	3.6	5.3
	With no depletion period		
	Fox Chow	3.0	7.8
V	" " + liver fraction	3.0	9.0
	Liver fraction + yeast (0.3 gm.)	13.2	7.8
	" " + " (0.6 " )	7.1	8.5
	" " + " (0.9 " )	6.1	10.2
VI	Control	2.6	5.1
	Liver fraction	17.4	6.4
	" " + rice polish concentrate (0.3 gm.)	4.8	9.3
	" " + " " " (0.6 " )	5.3	8.0
	" " + " " " (0.9 " )	2.2	7.3
VII	Control	2.6	5.1
	Liver fraction	17.4	6.4
	" " + lipocaic (0.3 gm.)	2.9	7.4
VIII	" " + lipocaic (0.025 gm.)	13.2	7.0
	" " + " (0.05 gm.)	13.9	7.0
	" " + " (0.1 gm.)	12.2	6.4
	" " + " (0.2 " )	5.6	6.1
	" " + " (0.3 " )	3.6	4.6
	" " + " (0.3 " )	3.2	6.1
IX	" " + lipocaic (0.3 gm.)	8.5	7.2
	" " + lipocaic (0.3 gm.)	3.2	6.8
	" " + casein (0.1 gm.)	8.1	6.4

All rats except those in Series III and IV received thiamine, choline, and riboflavin during the supplement period. In Series VIII vitamin B<sub>6</sub> was also administered.

caused by the liver fraction. It seemed possible that larger amounts of choline might be necessary to overcome the effects of the liver fraction and this was investigated in Series III.

*Series IV*—As choline, even in large amounts, did not prevent the development of fatty livers when the liver fraction was given, other substances were tested. Fox Chow (Toronto Elevators), regularly used as a diet for normal rats, was fed both after a 3 week depletion period on the basal diet and without a depletion period. This food, used in place of the basal diet, prevented the development of fatty livers when the liver fraction was given.

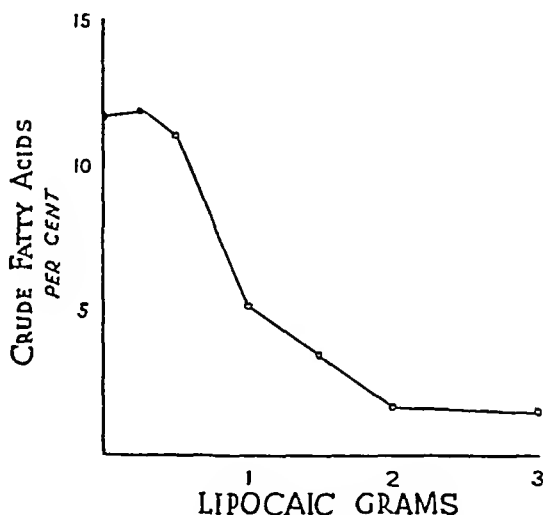


FIG. 1. Effects of various amounts of lipocaic upon liver fat

*Series V*—Dried brewers' yeast was tested for its activity at the levels indicated. Yeast is partially effective in controlling the level of liver fat but even 0.9 gm. of yeast per rat per day did not result in a normal value.

*Series VI*—A rice polish concentrate was tested. It was more effective than yeast in preventing the development of fatty livers when the liver fraction was given.

*Series VII*—Dragstedt and his colleagues (8) reported that when an alcoholic extract of pancreas, named by them lipocaic, was added to the diet of depancreatized dogs receiving insulin it prevented the infiltration of the liver with fat. It seemed of interest

to determine whether lipocaic would prevent the fatty livers caused by the liver fraction. This preparation proved to be by far the most active substance tested, on the basis of equal weights of the various preparations.

*Series VIII*—In the method reported by Dragstedt and his colleagues for the determination of the effectiveness of pancreatic extracts, depancreatized dogs have been used. This procedure is expensive and time-consuming. It seems likely that the prevention of fatty livers caused by liver extract in rats could be made the basis of a rapid procedure. Accordingly, in Series VIII the effect of various amounts of lipocaic was investigated. The results are given in Fig. 1. The potency of a pancreatic extract could be expressed in terms of the least amount required to maintain a normal liver fat in rats fed a constant amount of liver fraction, or the potency of various pancreatic extracts could be given in terms of a standard preparation.

*Series IX*—Besides a small amount of choline, which has been shown to be ineffective, lipocaic contains a relatively high percentage of protein (9). Several proteins, such as casein, have been shown to exert a lipotropic action upon the thiamine type of fatty liver (10). This series shows that extra casein did not exert the same effect as lipocaic.

#### *Effect of Liver and Pancreatic Extracts upon Cholesterol Metabolism*

Kaplan and Chaikoff (11) reported that in depancreatized dogs maintained with insulin there was an increase in fat content of the liver and also in cholesterol content, particularly in cholesterol esters. When raw pancreas was given, neither of these effects was observed, but the blood cholesterol was raised. This action of raw pancreas suggested the possibility that lipocaic might affect cholesterol metabolism.

*Series X*—By means of the Schoenheimer and Sperry technique (12), the amount of cholesterol in the crude fatty acid fraction of the liver and body was determined. A number of cholesterol determinations have been made and, in all series, two general trends have been in evidence. Administration of the liver fraction has caused increases in the amounts of cholesterol in the liver and in the body. Animals fed pancreatic extract have a small amount of cholesterol in the liver. The results of one series

only are given (Table III). Neither the basal diet nor the liver fraction contains cholesterol. It would seem likely that the increased amount was due to synthesis, since the quantities in both liver and body were increased. It will be noted that, under the conditions we have used, choline had no effect upon the amount of cholesterol in the liver, whereas lipocaic had. Rice polish concentrate and yeast also caused a reduction in liver cholesterol.

*Properties of Liver Constituent Influencing Fat Synthesis*

Preliminary work on the fractionation of the liver extract indicates that at least two constituents are involved in producing the

TABLE III  
*Alterations in Cholesterol Content*

Supplements	Total cholesterol in		Total crude fatty acids in	
	Liver	Body	Liver	Body
	mg.	mg.	mg.	mg.
None, 3 wks. depletion.....	1	35	56	798
Control.....	7	62	119	2213
Liver fraction .....	40	91	786	3969
" " + lipocaic (0.3 gm.)..	15	108	178	5130
" " + choline (50 mg.)..	44	77	799	3393

All rats received thiamine, choline, riboflavin, and vitamin B<sub>6</sub> during the supplement period.

changes in amount of fat and in body weight. The factor responsible for production of fatty livers is heat-labile, can be adsorbed on fullers' earth and eluted with hot ethyl alcohol, is soluble in water, ethyl alcohol, acetone, and insoluble in amyl alcohol. The growth factor is similarly adsorbed and has the same solubilities but is heat-stable.

DISCUSSION

The feeding of a liver fraction to rats on a fat-free diet causes a marked synthesis of fat, as evidenced by an increase in both liver and body fat. The failure of large doses of choline to prevent the fatty liver caused by administration of the liver fraction suggests



that the character of the fat in the liver is different from that found in the fatty liver caused by feeding a diet containing thiamine but low in choline; it has been shown by one of us (13) that the thiamine type of fatty liver is easily prevented or cured by small doses of choline. We are unable at present to describe the type of fat present in the fatty liver produced by feeding the liver fraction other than to note that it contains an increased amount of cholesterol.

The action of lipocaic in preventing fatty livers of this special type indicates that it contains a lipotropic factor other than choline. A similar effect was caused by rice polish concentrate and by brewers' yeast but evidence is not available that the same factor is responsible in each case. It is now clear that at least three kinds of alimentary fatty liver can be produced: by feeding a thiamine-rich, low choline diet; by feeding cholesterol; and by feeding a liver extract. The different responses of these fatty livers to various lipotropic agents may explain the apparently discrepant results which have been reported with regard to the action of lipocaic. The choline and protein present in the lipocaic preparation which we have used would account for its lipotropic action on the thiamine type of fatty liver, as has been shown by Best and Ridout (9), but these constituents are not responsible for the effects which we have observed.

While the type of fatty liver caused by liver extract is prevented by lipocaic, we are not in a position to say that it is similar to the fatty liver found in depancreatized dogs, nor that the effects of lipocaic in the two cases are due to the same constituent. The results secured with rice polish concentrate and yeast suggest that the lipotropic activity of pancreatic extract which we have observed is not due to a hormone. The lipotropic effect is associated with, or due to, a reduction in liver cholesterol.

Blatherwick and associates (14) reported the production of fatty livers in rats by the feeding of whole liver or of several liver fractions. In their investigations the amounts of cholesterol esters and of neutral fat in the livers were increased. When whole liver was fed, the increase in cholesterol esters may have been caused partly by the ingestion of the cholesterol contained in the liver, as these workers pointed out. That explanation was not likely

to be true for some of the fractions which they tested and they concluded that beef liver contains a water-soluble substance which causes fatty livers when fed to rats. While they did not use a fraction similar to that which we have employed, our results definitely support their conclusion. The fraction which we have used contains no cholesterol. Blatherwick *et al.* found that the administration of large amounts of choline failed to prevent the fatty livers; our results in this respect also are in complete agreement. However, in our experiments, the fatty livers resulted from a synthesis of fat, whereas dietary fat was supplied in the experiments of Blatherwick and associates.

Recently Engel and Phillips (15) have described the production of fatty livers in rats by thiamine administration. The basal diet used by them contained a liver fraction as a source of factor W. The fatty livers which they reported were similar to those found in the present investigation in that they were not prevented by choline. It seems likely that Engel and Phillips would have secured the thiamine type of fatty liver (preventable by choline) if the liver fraction had been omitted from the basal diet.

It is not possible at present to draw any conclusions regarding the constituents of the liver fraction which cause fatty livers by increasing the synthesis of fat. The fraction contains many substances other than pantothenic acid and factor W. Preliminary results secured with preparations from the liver fraction suggest that at least two constituents may be responsible for the observed effects. The effect of the fraction upon the body weights of rats was comparable to that secured by Elvehjem, Koehn, and Oleson (5) in their studies of factor W, but chemical evidence indicates that factor W may not be concerned.

#### SUMMARY

The administration of a crude liver fraction to rats causes marked synthesis of fat and the development of fatty livers highly resistant to the lipotropic action of choline. There are coincident increases in the amounts of cholesterol in the liver and body, apparently as a result of synthesis. The increase in fat and cholesterol in the liver can be prevented by feeding a pancreatic extract (lipocaic), rice polish concentrate, or brewers' yeast.

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## FATTY ACIDS SYNTHESIZED BY THE ACTION OF THIAMINE\*

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Fat which has been synthesized by rats from carbohydrates is characterized by the presence of large amounts of  $C_{16}$  fatty acids (1). In such synthetic depot fat, the total  $C_{16}$  acids constitute 40 to 45 per cent of the total mixed acids. The value for the  $C_{16}$  acids is approximately 25 per cent when fat is present in the animal's diet and is being deposited directly. A particularly striking increase is noticed in the proportion of palmitoleic ( $\Delta^9,^{10}$ -hexadecenoic) acid in the synthetic fat. On a molar basis, the values for this component are usually about 15 per cent in the synthetic fat, compared with 3 to 4 per cent when the animal is ingesting fat rather than synthesizing its depot fat entirely from non-lipid sources.

The production of fat from carbohydrate in the presence of thiamine was demonstrated by McHenry (2) who showed a marked increase in liver fat in young rats when the vitamin was added to a basal diet low in choline and fat-free. Subsequent studies (3) showed that thiamine, as a supplement to basal rations which were deficient in vitamin  $B_1$ , caused an increase in the amount of total fatty acids in both rats and pigeons. These results strongly supported the hypothesis advanced by McHenry (4) that "vitamin  $B_1$  is necessary for the synthesis of fat from carbohydrate."

The present test of the above hypothesis of thiamine action was

\* A preliminary report of these data was presented at the meeting of the American Chemical Society at Cincinnati, April, 1940. The investigation was aided by a grant from the Buhl Foundation.

suggested by the earlier evidence that synthetic fat may be characterized by the quantitative determination of the component fatty acids. If thiamine is required for the synthesis of fat from carbohydrate, then the high amounts of  $C_{16}$  acids characteristic of synthetic fat should not be found when animals ingest a high carbohydrate ration containing neither fat nor a source of thiamine, but they should appear quickly following thiamine administration.

#### EXPERIMENTAL

Young albino rats of the Wistar strain reared in the Connaught Laboratories were employed. They were fed a stock ration (Toronto Elevators Company Fox Chow) from weaning to the start of experimental feeding. When they had grown to an average weight of 88 gm., they were fed a diet free of fat and without the vitamin B complex or choline. It was low in casein to reduce the lipotropic effect similar to that of choline. The ration had the following composition, in per cent by weight, casein 10, cane sugar 84, salt mixture (Steenbock-Nelson, Salts 40 (5)) 4, agar-agar 2, cod liver oil concentrate 0.015.

98 rats were maintained on this diet for 20 days. At the end of this time when the average body weight was 68 gm. 50 of the animals were killed by stunning and the remaining forty-eight rats were continued on the basal ration supplemented with 12.5 micrograms of thiamine chloride (Merck) per rat per day for 12 days and then killed. During these 12 days the average weight increased to 74 gm. The livers from each group were immediately removed and placed in acetone. The other organs and the complete gastrointestinal tract were removed from each carcass. The various lipid fractions desired for analysis were obtained from the livers and the finely ground carcasses by methods described previously (1). Tables I and II show the amounts and general characteristics of the resulting material.

In an experiment carried out similarly to the above it was found that the maintenance of rats on the basal ration for 4 weeks caused a depletion of fat coincident with the decrease in body weight. The iodine number of the total fatty acids of the visceral fat was greater than the value found at the beginning of the experiment. The changes in body weight, liver and body fat,

and in iodine numbers are given in Table III. It should be noted that such increases in unsaturation of the acetone-soluble lipids were not obtained in earlier studies when body fat was depleted by fasting (1, 7).

TABLE I  
*Amounts of Lipids Obtained*

Source and type of lipid	Vitamin B complex-deficient animals (50)			Thiamine-treated animals (48)		
	gm.	gm. per 100 gm. rat	gm. per 100 gm. liter	gm.	gm. per 100 gm. rat	gm. per 100 gm. liter
Carcass						
Total lipids.....	52.41	1.54		121.30	3.42	
Acetone-soluble lipids....	41.94	1.23		110.91	3.12	
Liver						
Total lipids.....	4.84		3.10	16.59		8.90
" fatty acids.....	3.40		2.18	14.22		7.60

TABLE II  
*General Characteristics of Depot and Liver Lipids*

Lipids	Saponification equivalent	Iodine No.*	Lead salt separation of mixed fatty acids			
			Solid acids		Liquid acids	
			gm.	per cent	gm.	per cent
Depot acetone-soluble lipids						
Vitamin B complex-deficient group.....	319.0	74.9	8.40	25.0	25.23	75.0
Thiamine-treated animals....	292.7	60.0	29.41	32.8	60.28	67.2
Total liver lipids						
Vitamin B complex-deficient group.....	308.2	79.4	0.89	30.2	2.06	69.8
Thiamine-treated animals....	289.5	64.2	4.80	35.3	8.81	64.7

\* Rosenmund-Kuhnenn method (6).

The fatty acids from the acetone-soluble portion of the body lipids and the total acids from the liver lipids were both qualitatively and quantitatively analyzed by the ester distillation procedure. Initial resolution of the mixed acids as soluble and insoluble lead salts in alcohol was accomplished by a modified Twitchell method (*cf.* (8)). Neutral methyl esters were pre-

TABLE III  
*Body and Liver Fat Changes during Depletion Period*

Duration of depletion period	Average body weight (10 animals)	Total fatty acids		Iodine No. of total fatty acids	
		Liver	Body	Liver	Body
days	gm.	gm. per 100 gm. liver	gm. per 100 gm. rat		
At start	104	3.4	7.7	90	72
7	93	4.6	5.4	73	68
14	84	5.1	4.0	86	78
21	75	4.9	2.4	103	86
28	62	2.6	1.6	104	94

TABLE IV  
*Composition of Fatty Acids of Rat Carcass Glycerides and Liver*

Acids	Carcass glycerides				Liver			
	Vitamin B complex-deficient animals		Thiamine-treated animals		Vitamin B complex-deficient animals		Thiamine-treated animals	
	weight per cent	molar per cent	weight per cent	molar per cent	weight per cent	molar per cent	weight per cent	molar per cent
Myristic.....	2.7	3.2	0.9	1.0	1	1	1	1
Palmitic.....	20.6	21.9	27.4	29.6	18	20	27	29
Stearic.....	6.2	6.0	3.5	3.3	3	3	3	3
Arachidic.....	2.6	2.3	2.3	1.9	3	3	1	1
Tetradecenoic.....	1.2	1.5	0.5	0.6				
Hexadecenoic.....	4.9	5.3	10.5	11.1	5	5	14	15
Oleic.....	54.3	52.5	50.9	48.7	50	49	48	46
Linoleic.....	5.6	5.6	3.2	3.1	8	8	2	2
Arachidonic.....	1.9	1.7	0.8	0.7	12	11	4	3
Totals								
C <sub>14</sub> .....	3.9	4.7	1.4	1.6	1	1	1	1
C <sub>16</sub> .....	25.5	27.2	37.9	40.7	23	25	40	44
C <sub>18</sub> .....	66.1	64.1	57.6	55.1	61	60	53	51
C <sub>20</sub> .....	4.5	4.0	3.1	2.6	15	14	5	4
Saturated.....	32.1	33.4	34.1	35.8	25	27	32	34
Unsaturated.....	67.9	66.6	65.9	64.2	75	73	68	66

pared and fractionally distilled through Column 15-Ag as described previously (1). As usual, the quantitative composition of the various ester fractions was established by direct evidence

(the formation of characteristic derivatives or the isolation of the acid itself) except in the case of the tetra- and hexadecenoic acids. Indirect evidence for the occurrence of the  $C_{14}$  and  $C_{16}$  monounsaturated acids was obtained by oxidizing ester fractions suspected from their equivalent weights and iodine numbers to contain the  $C_{14}$  and  $C_{16}$  saturated and unsaturated components (9). In each case, the unoxidized saturated esters were found to have almost exactly the same equivalent weight as the original ester mixture taken for oxidation. It follows that the unsaturated esters must have had the same carbon chain length as the saturated esters, namely  $C_{14}$  and  $C_{16}$ . The quantitative composition of each ester fraction was calculated from the weights of the individual fractions and their respective analytical constants, saponification equivalent and iodine number. From these values and the percentage of acids obtained as "solids" and "liquids" in the lead salt separation of the mixed acids, the percentage composition of the mixed acids follows directly. The distribution of acids in the glyceride (acetone-soluble) portion of the depot lipids and the values for the total liver acids are given in Table IV.

#### DISCUSSION

The data presented above furnish considerable evidence that fat synthesis resulted as a direct effect of feeding thiamine. Body stores of fat were utilized, apparently without synthesis, during the depletion period when animals were maintained on a high carbohydrate diet lacking in the B vitamins. Fat synthesis followed the thiamine administration immediately, however, as was indicated by the rise in both the body and liver fat and the marked change in fatty acid composition. The high amount of  $C_{16}$  acids (40.7 per cent) previously noted as characteristic of synthetic fat provides unmistakable evidence of the process stimulated by thiamine.

During the period when thiamine was fed, forty-eight animals gained an average of 6 gm. The acetone-soluble portion of the depot lipids accounted for approximately 25 per cent of this increase (1.47 gm. per rat more in the thiamine-treated group than in the non-treated group). In terms of total fatty acids per 100 gm. of rat body weight, the gain was 1.81 gm. The absolute increase for the major component fatty acids may be obtained



by difference from the values recorded in Table V. The figures in this table were calculated from the data given in Tables I, II, and III for the acetone-soluble lipids, their equivalent weights being corrected for the unsaponifiable material and the percentage composition by weight of the respective fatty acids.

The fatty acid analyses show a marked difference in composition between the body fat from the deficient animals and those subsequently given thiamine. All of the acids changed somewhat in proportion but the significant change appears in the  $C_{16}$  acids, palmitic and hexadecenoic acids. Together, the molar ratios of these acids have been raised from 27.2 to 40.7 per cent with the thiamine treatment. This is regarded as evidence that fat was

TABLE V

*Major Fatty Acids in Carcass Glycerides per 100 Gm. of Rat Body Weight*

Acids	Vitamin B complex-deficient group (equivalent weight of glycerides = 290.5)	Thiamine-treated animals (equivalent weight of glycerides = 281.8)
	gm.	gm.
Total acids.....	1.06	2.87
Palmitic.....	0.22	0.79
Hexadecenoic.....	0.05	0.30
Oleic.....	0.58	1.46

synthesized only when thiamine was present in the diet. It would appear that the  $C_{16}$  fatty acids are involved either as intermediates or end-products in the biological synthesis of fat from carbohydrate or protein and that thiamine is an agent capable of bringing about intermediate stages in such conversions. The close similarity of the liver fatty acids with those present in the depots may mean that the liver is the seat of such transformations *in vivo*. This view is substantiated by the proportionately greater increase in liver fat, as compared with the increase in body fat. The widespread occurrence in nature of  $C_{18}$  fatty acids, and indeed the synthesis of them in the experiments reported here, makes it necessary that any conception of the mechanism of fat synthesis from non-lipid sources include them.

## SUMMARY

The molecular distribution of the fatty acids in body and liver fat of thiamine-deficient and thiamine-treated rats has been studied.

When young rats were taken from a ration containing fat (Fox Chow) and fed for 3 weeks on a vitamin B complex-deficient ration which was free of fat, they lost fat gradually from their body stores. An increase in the iodine numbers of the total lipid fatty acids but not in the acetone-soluble lipids in both the body and liver was observed during the depletion period. Addition of 12.5 micrograms of thiamine daily to the basal ration during a subsequent 12 day period caused a rapid deposition of fat in the body. The liver fat was also markedly increased owing to the absence of dietary choline. The total acetone-soluble lipids of a group of forty-eight young rats increased from 40.3 to 110.9 gm. following thiamine treatment. Expressed in terms of fatty acids per 100 gm. of body weight, the increase due to thiamine was 1.81 gm. of which 49 per cent was due to oleic acid, 32 per cent to palmitic acid, and 14 per cent to hexadecenoic acid. The synthetic fat was characterized by an increase in the  $C_{16}$  acids from 25 to 41 per cent (molar basis) of the total mixed acids.

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# THE PREPARATION OF CELLOPHANE MEMBRANES OF GRADED PERMEABILITY

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In studies of osmotic pressure of colloidal solutions, and in the determination of molecular size by filtration, it is necessary to use membranes of known porosity, and desirable to be able to obtain such membranes regularly and cheaply. Cellophane as formerly made was permeable to small quantities of serum protein, but the present product is not. However, McBain and Steuwer (1) found that immersion of cellophane in zinc chloride solutions of varying concentration would alter its permeability, and they studied the effect of dilution of electrolytes, varying pressure, etc., on ultrafiltration. In the present work this effect has been re-studied and extended by calculation of the pore size of the membranes obtained, and the filtration patterns of certain protein and carbohydrate materials have been determined.

## *Materials and Methods*

Plain transparent No. 300 cellophane was immersed in zinc chloride solutions (90 to 120 gm. per 100 cc. of solution) for 15 minutes at 25°. The zinc chloride solution was then decanted, and the membrane washed in running tap water for about 30 minutes. After it was washed, no zinc chloride remained.

The ultrafilters were made of brass, and coated internally with paraffin. The membrane was supported on several layers of filter paper. Distilled water was used to determine rate of flow, measured as the volume filtered in a measured time, under constant pressure. Temperature was noted at each observation and viscosity determined from standard tables.

Membrane thickness was determined by an apparatus similar

to that described by Krogh (2), with a calibrated ocular micrometer. Ten readings were taken on each membrane, and the mean computed.

The water content of the membranes was determined by weighing a portion of known area both in the wet state and after drying for 24 hours at approximately 25° (the humidity being approximately 30 per cent).

Crystalline egg albumin was prepared by the method of Hopkins and Pinkus (3), hemoglobin by the method of Bourdillon (4). Proteins were determined in triplicate by Howe's method (5), hemoglobin photometrically by Heilmeyer's method (6). Sodium arabinatate was determined by precipitating it with ferric chloride and ethyl alcohol and drying to constant weight.

### *Measurement of Porosity*

Several investigators (7-10) have shown that collodion and cellophane membranes behave like a series of small capillaries when water is allowed to flow through them; in other words, their porosity may be calculated by the application of Poiseuille's law, which Elford (11) has expressed as follows:

$$r = 2l \sqrt{2qn/pv}$$

when  $r$  is the pore radius in cm. (usually expressed in  $m\mu$ ),  $l$  the thickness of membrane in cm.,  $q$  the rate of flow of water (cc. per sq.cm. per second),  $n$  the coefficient of viscosity of water,  $p$  the pressure applied in dynes per sq.cm., and  $v$  the total pore volume ( $\approx$  difference between wet and dry weights of membrane per sq.cm.).

Fig. 1 shows the calculated average pore diameter of cellophane membranes treated with various concentrations of zinc chloride solutions under constant conditions of temperature. A similar range of porosity may be produced by using a constant concentration of zinc chloride solution but varying temperatures, the porosity increasing as the temperature increases.

The figures obtained from the application of Poiseuille's law cannot be regarded as absolute for several reasons: (1) the pores of altered cellulose are probably slit-like in cross-section rather than circular (12); (2) the capillary length,  $l$ , is assumed to be

equal to the thickness of the membrane, whereas it is probably longer; (3) the total volume of pores is taken to be equivalent to the water content, but this assumes that there are no blind or intersecting passages. However, the law does provide a means of estimating the relative permeability of membranes and the relative size of colloidal molecules, and experience has shown that these factors do not introduce a very large element of error.

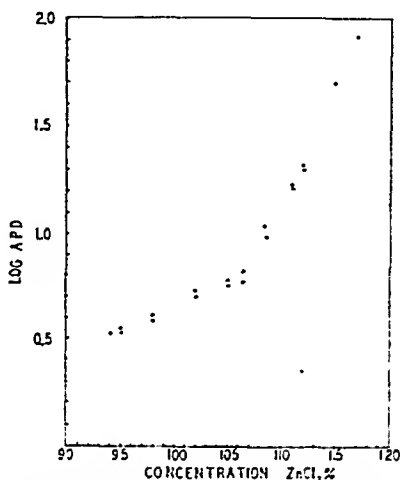


FIG. 1. Calculated average pore diameter (APD) in  $m\mu$  of membranes treated in zinc chloride solutions of varying concentration. Pressure applied, 500 mm. of mercury; water temperature  $25^\circ$ ; zinc chloride solutions at  $25^\circ$ .

### *Physical Alterations and Properties of Membranes Treated with Zinc Chloride Solutions*

**Thickness**—No. 300 cellophane swollen in water has a thickness of approximately 0.004 cm. Soaking it in increasing concentrations of zinc chloride solutions causes an increase in the thickness of the membrane, the most porous (pore diameter  $80 m\mu$ ) being 0.0195 cm. thick.

**Water Content**—The ratio of the difference between wet and dry weights of a membrane and its wet weight has been termed specific water content by Elford and Ferry (13), and ideally should approach 1.0. Altered cellophane membranes have a

specific water content of 0.65 to 0.94, whereas collodion membranes within a similar range of porosity have a specific water content of 0.41 to 0.87 (Elford and Ferry). The higher the specific water content, the less possibility is there for the existence of blind and non-intersecting pores.

The altered cellophane membranes appear to dry merely by losing water from the interstices, since the rate of drying is proportional to the original water content. Table I shows measurements made on a series of membranes of similar area but varying porosity; the ratio between time of drying and total water content is fairly constant.

*Filtration Pressure*—The rate of flow of water was proportional to the pressure used, between 100 and 500 mm. of mercury. Pres-

TABLE I  
*Water Content of Membranes of Similar Area but Varying Porosity*

Total water content	Time of drying	$\frac{\text{Time}}{\text{Water content}}$
<i>mg.</i>	<i>min.</i>	
170	34	0.200
404	88	0.218
435	86	0.198
1257	228	0.181

ures must be of this magnitude in the smaller porosity ranges in order to secure an adequate volume of filtrate. No differences in the thickness of the membranes were noted before and after filtration.

*Adsorption of Protein*—Hitchcock (14) showed that collodion membranes adsorb weighable amounts of egg albumin. A number of membranes of 30 m $\mu$  porosity and 50 sq.cm. area were immersed in solutions of 1.45 per cent crystalline egg albumin at isoelectric pH, and in normal human serum; after 24 hours the membranes were removed, washed with distilled water, and the total nitrogen determined by the Kjeldahl method. The maximum amount found in any membrane was 0.51 mg. of protein, a negligible quantity.

*Autoclaving*—Autoclaving for 20 minutes at 15 pounds pressure at 120° causes essentially no change in porosity.

TABLE II  
Filtration Patterns of Colloidal Solutions

Experiment I. Crystalline egg albumin; 1 per cent solution at pH 5.70; pressure, 500 mm. Hg; effective area of filter, 19.7 sq.cm.

Pore diameter	Per cent of original concentration in ultrafiltrate	Approximate time necessary to attain constant concentration in ultrafiltrate
mm		min.
2.98	0	
4.03	68	110
5.47	100	80

Experiment II. Hemoglobin; 0.5 per cent solution at pH 6.60; pressure, 500 mm. Hg; effective area of filter, 19.7 sq.cm.

3.28	0	
5.90	52	120
8.10	100	80

Experiment III. Human serum; diluted 1:7 in 0.85 per cent saline; no observations recorded concerning filtration time

Pore diameter	Per cent of original concentration in ultrafiltrate	
	Albumin	Globulin
mm		
4.23	12.6	0
5.44	31.5	0
5.66	40.0	18
12.00	56.0	47
17.07	55.0	65

Experiment IV. Sodium arabinat; used to ascertain effects of changes of pH and electrolyte concentration on filtrability; concentration of gum, 3.9 gm. per cent; membrane porosity, 17 mm; effective area of filter, 99.4 sq.cm.; pressure, 500 mm. Hg

Solution No.	Per cent of original concentration in ultrafiltrate	Approximate time necessary to attain constant concentration in ultrafiltrate
		min.
1. Water, pH 4.23	2	14
"    "    5.35	13	17
"    "    11.60	0	
2. 2% NaCl, pH 4.08	18	17
2%    "    "    4.68	20	15
5%    "    "    6.70	46	13



*Reproducibility*—Standard solutions of zinc chloride at constant temperature will produce membranes of known porosity, up to 12 m $\mu$ ; there is some variation in porosity with pore sizes greater than this.

*Storage*—Membranes after calibration can be kept unchanged indefinitely if soaked in 50 per cent glycerol and dried (McBain and Steuwer).

### *Filtration Behavior of Selected Colloids*

Ferry (15) pointed out that an ultrafiltrate may be less concentrated than the original solution because (a) the disperse phase may be adsorbed; (b) the disperse phase may block the pores; (c) the larger particles of the disperse phase may be mechanically retained—sieving. The sieving effect is the desired objective in ultrafiltration. Furthermore, when a colloid is being filtered, the concentration of the disperse phase in the ultrafiltrate must attain a constant level for the result to be acceptable. Optimal conditions for ultrafiltration are obtained when the concentration of the disperse phase is high, the total pore area relatively low, and isoelectric pH is avoided (Ferry). These factors all tend to lessen adsorption, a potent factor in the use of collodion membranes but probably negligible with cellophane.

Filtration patterns of a number of colloidal solutions were determined. Protocols of a number of typical experiments are presented in Table II.

### SUMMARY

A method is described for the preparation of cellophane membranes of predictable pore size. It was found that the difference in pore size of membranes which allowed no passage of egg albumin and those which allowed complete filtration was very small. The same statement applies to hemoglobin solutions. This fact, according to Ferry, is an excellent criterion both for membrane isoporosity and molecular homodispersion. On the other hand, the range of pore sizes required for the filtration of the proteins of human serum was quite large, the data supporting the conception that human serum protein is a heterodisperse system. Changes in pH and in salt concentration were found to affect the filtrability of sodium arabinates markedly.

Cellophane membranes of graded porosity can easily and quickly be prepared by the use of zinc chloride solutions. These membranes, which are isoporous and reproducible, are suitable for ultrafiltration and fractional ultrafiltration of solutions of small colloidal molecules.

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# INFLUENCE OF IODOACETIC ACID ON SULFUR METABOLISM. GROWTH STUDIES IN THE YOUNG RAT\*

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The extensive experimental use of iodoacetic acid for altering normal reactions has disclosed suggestive relationships between this compound and certain substances containing the sulfhydryl group. Iodoacetic acid inhibition of methylglyoxalase is reversed by the tripeptide glutathione, or by cysteine (1); the poisoning effect of iodoacetic acid on the carbohydrate metabolism of brain tissue can be almost completely prevented by the addition of glutathione or cysteine (2); inhibition of development of sea urchin eggs, produced by iodoacetic acid, may be overcome and growth reinitiated under the influence of sulfhydryl (3). These experiments and many others strongly suggest that a portion of the effect produced by iodoacetic acid in many systems may be explained on the basis of a combination of the halogen-containing acid with sulfhydryl, a reaction which has been demonstrated to occur *in vitro* (1, 3, 4) and which may be represented as follows:

$$\text{R-SH} + \text{ICH}_2\text{COOH} \rightarrow \text{R-SCH}_2\text{COOH} + \text{HI}.$$

In view of the evidence suggesting a reaction between sulfhydryl and iodoacetic acid, the present study was initiated to examine the influence of iodoacetic acid on the reactions in the living organism which involve sulfur-containing compounds. It has previously

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A preliminary report was presented before the American Society of Biological Chemists (Simon, E. E., and White, A., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, 123, p. cix (1938)).

† The data of this paper are taken from a portion of a dissertation presented by Elizabeth Simon Stevenson as partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1939.

been demonstrated that a wide variety of organic compounds inhibits the growth of young rats subsisting on a diet of relatively low content of sulfur-containing amino acids (5, 6). The data obtained have led to the suggestion that these growth-inhibiting substances exert their effect by imposing on the organism an abnormally high demand for the sulfur-containing amino acids for detoxication mechanisms. Thus the organism is deprived of essential elements required for the synthesis of the new tissue necessary for growth.

With a similar experimental approach, a study has been made of the effect of iodoacetic acid on the growth of the rat. The influence of various supplements on the inhibited growth rate has been determined.

#### EXPERIMENTAL

Male rats, at weaning, were placed in individual cages and fed, *ad libitum*, a basal diet having the following composition: casein<sup>1</sup> 6, starch 50, lard 24, sucrose 15, cod liver oil 1, and salt mixture<sup>2</sup> 4 per cent. In addition, each animal received a daily supplement of 400 mg. of dried yeast.<sup>3</sup> When the animals had reached a body weight of approximately 75 to 85 gm., 100 mg. of iodoacetic acid<sup>4</sup> were incorporated into each 100 gm. of the basal diet. Growth was immediately inhibited. After the animals had subsisted for at least 4 weeks on the iodoacetic acid-containing diet, a supplementary compound was incorporated into the ration already containing iodoacetic acid. The supplementary compounds and the amounts of each incorporated singly into each 100 gm. of the iodoacetic acid-containing basal diet were *l*-cystine (360 mg.), *l*-cysteine hydrochloride (470 mg.), *dl*-methionine (450 mg.), *dl*-homocystine (402 mg.), *d*-cystine (360 mg.), taurine (375 mg.), anhydrous sodium sulfate (426 mg.), and *l*-phenyluraminocysteine (717 mg.). The *l*-cystine was obtained from hair; *l*-cysteine hydrochloride was prepared by reduction of *l*-cystine (8); *dl*-methionine was obtained from the Organic Chemical Manufactures Division,

<sup>1</sup> Casein No. 453, Casein Company of America.

<sup>2</sup> Osborne and Mendel salt mixture (7).

<sup>3</sup> Northwestern Yeast Company.

<sup>4</sup> Eastman Kodak Company product, recrystallized from warm petroleum ether until a melting point of 81–82° (uncorrected) was obtained.

University of Illinois. The *dl*-homocystine was prepared from *dl*-methionine by the method of Butz and du Vigneaud (9), with the modification suggested by Brand, Cahill, and Block (10). Optically pure *d*-cystine was obtained by resolution of *dl*-cystine (11); taurine was prepared by decarboxylation of cysteic acid (12); *l*-phenyluraminocysteine was obtained by the reduction of *l*-diphenyluraminocystine with thioglycolic acid, with subsequent purification by repeated precipitation from ethyl acetate solution by the addition of 5 volumes of petroleum ether. Analysis for sulfhydryl sulfur indicated that 75 per cent of the isolated product was in the reduced form.

A second group of supplements was injected subcutaneously into animals ingesting the growth-inhibiting, iodoacetic acid-containing basal diet. Solutions of *l*-cysteine hydrochloride (38 mg. per rat per day), *dl*-methionine (36 mg. per rat per day), and glutathione (56 mg. per rat per day) were made immediately prior to injection, each solution being adjusted to approximately pH 7 with 0.1 *N* sodium hydroxide. Solutions of thioglycerol, riboflavin, and riboflavinphosphoric acid were made in quantities sufficient for not more than 1 week. 20 mg. of thioglycerol, and 40  $\gamma$  each of riboflavin and of riboflavinphosphoric acid were injected daily. Glutathione was obtained from Hoffmann-La Roche, Inc.; thioglycerol (60 per cent solution in glycerol) was kindly furnished by the Abbott Laboratories, Chicago. Riboflavin was obtained from the S. M. A. Corporation, Cleveland. Riboflavinphosphoric acid was synthesized from riboflavin by the method of Kuhn and Rudy (13). All of the compounds employed were analytically pure. The required quantity of each of the injected supplements mentioned above was contained in an administered volume of 0.5 cc. The effect of cortin supplements was determined by injection of 1 cc. of a cortin solution (Upjohn) three times daily. The quantity of cortin administered is sufficient to maintain an adrenalectomized animal in a healthy condition.

### Results

A summary of the average daily weight changes and food consumptions under the various dietary conditions of the study is given in Table I and representative graphs illustrating the

growth rates of the animals are presented in Figs. 1 to 3. The data show the inhibitory effect on growth resulting in young rats weighing about 80 gm. when iodoacetic acid is incorporated into

TABLE I

*Summary of Average Weight Change and Average Food Consumption of Animals Receiving Basal Diet and Various Types of Supplements*

Diet	Designation of diet in Figs. 1 to 3	No. of animals in group	Average daily weight change	Average daily food consumption
			gm.	gm.
Basal	C-6	83	+1.8	6.8
" + iodoacetic acid	I	95	0.0	3.6
" + injected iodoacetic acid	I'	8	+0.3	5.3
" + " " " + injected cysteine	I'-CH'	3	+1.5	5.7
Iodoacetic acid-containing basal diet + each of the following growth-promoting supplements				
Dietary <i>l</i> -cystine	I-IC	14	+1.0	5.5
" <i>l</i> -cysteine hydrochloride	I-CH	7	+1.3	7.2
Injected "	I-CH'	17	+1.0	5.8
Dietary <i>dl</i> -methionine	I-M	9	+1.1	5.4
Injected "	I-M'	8	+0.7	5.8
Dietary <i>dl</i> -homocystine	I-H	4	+1.5	6.3
Injected glutathione	I-GSH'	8	+1.0	5.1
Iodoacetic acid-containing basal diet + each of the following ineffective supplements				
Dietary <i>d</i> -cystine	I-dC	5	-0.2	4.2
" taurine	I-T	5	+0.1	4.3
" sodium sulfate	I-S	4	-0.4	4.0
" <i>l</i> -phenyluraminocystine	I-PC	7	-0.2	4.2
Injected thioglycerol	I-TG'	5	+0.2	4.1
" riboflavin	I-F'	3	-0.05	4.0
" riboflavinphosphoric acid	I-FP'	6	-0.13	4.1
" cortical extract	I-CE'	4	0.0	4.1

the basal diet. This observation is similar to the finding of Laszt and Verzár (14); the latter investigators succeeded in arresting growth by the inclusion of iodoacetic acid at a level of 1 part in 5000 parts of diet. The addition of *l*-cystine to the basal diet

containing iodoacetic acid results in a marked stimulation of growth as well as an increase in food consumption. The growth-

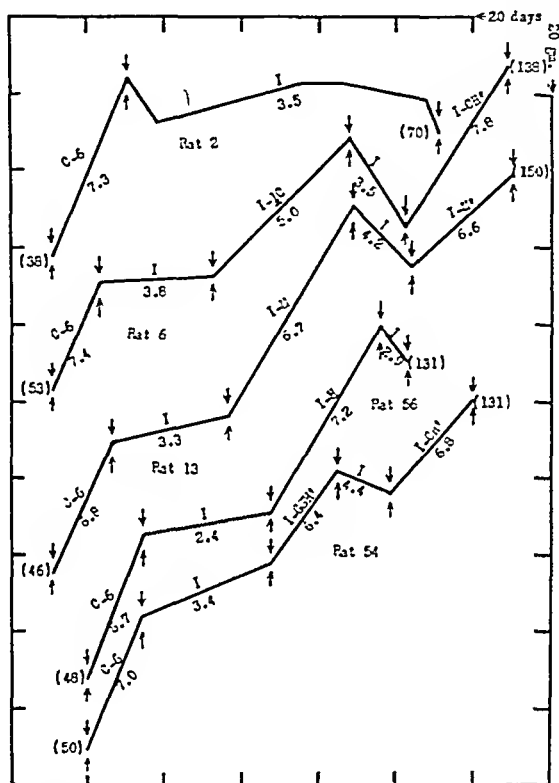


FIG. 1. Typical growth results obtained on basal diet, on basal diet with added iodoacetic acid, and on iodoacetic acid-containing basal diet as influenced by various supplements. The diet employed in any portion of an experiment is indicated between two downward arrows representing the beginning and end of a period. For interpretation of the abbreviations designating the diets in various periods, see the second column of Table I. The quantities of the supplements administered are given in the text. The average daily food consumption in gm. for the corresponding interval is shown by the figures between the upward arrows. The initial and final body weights are presented in parentheses.

stimulating ability of *l*-cystine in animals fed iodoacetic acid is strikingly similar to previous studies (5, 6) in which this amino





the injected amino acid stimulated growth as well as did dietary cystine, it is concluded that the growth-promoting action of cystine supplements is not dependent on a preliminary combination of iodoacetic acid with cystine prior to the absorption of these compounds. In other experiments, iodoacetic acid and l-cysteine were injected subcutaneously on opposite sides of the

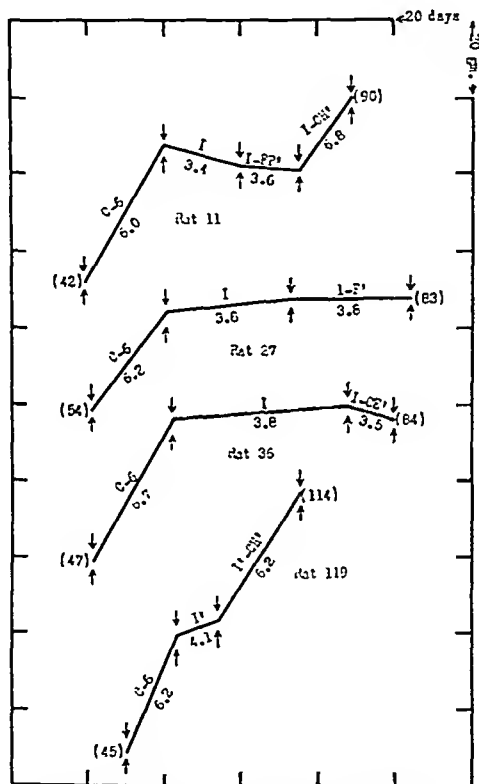


Fig. 3. For the dietary abbreviations used in this chart, see the second column of Table I. The other designations are the same as in Fig. 1.

animal further to demonstrate that the intestine, or an intestinal factor, does not detoxicate iodoacetic acid before absorption. The data in these instances were similar to those obtained with orally administered supplements. Young rats exhibit an extreme sensitivity to injected iodoacetic acid, a fact previously noted by Genevois and Brisou (15). After a considerable number of

trials, it was found that the daily subcutaneous injection of an aqueous solution containing 0.4 mg. of iodoacetic acid, neutralized to pH 7.0 with 0.1 N sodium hydroxide, produced a growth inhibition similar in extent to that observed with orally administered iodoacetic acid (1 part in 1000 parts of the diet). Cysteine injections under these circumstances caused a prompt resumption in growth, despite continued simultaneous injection of the halogen-containing fatty acid. It may be mentioned that rats obtained from different colonies, the Yale laboratory and the Connecticut Agricultural Experiment Station, exhibited striking differences in their ability to tolerate the toxic effects of injected iodoacetic acid. Young rats from the Connecticut Agricultural Experiment Station could not be employed for studies of the effects of injected iodoacetic acid, inasmuch as their growth was not inhibited to any appreciable extent by the quantities of iodoacetic acid used to stunt animals in the laboratory colony.

Further suggestive evidence indicating that the relationship between iodoacetic acid and cystine is metabolic in nature is seen in the restricted pathological changes found on histological examination.<sup>6</sup> The lungs, intestine, adrenals, pancreas, and spleen of iodoacetic acid-fed animals exhibited no abnormalities. However, in the four animals examined in detail, there was present marked necrosis of the convoluted tubules of the kidneys; destruction of the cellular structure had occurred with no evidence of regeneration. The localization of the pathological symptoms emphasizes the fact that the effects of iodoacetic acid observed in the present study cannot be accounted for by wide-spread toxic symptoms in the animal organism.

The sulfur-containing amino acids *dl*-methionine, *dl*-homocystine, and *l*-cysteine have all been demonstrated to substitute for *l*-cystine in the conventional cystine-low diets (16-18). Further, methionine functions in lieu of cystine in promoting growth in rats stunted by a wide variety of toxic, organic compounds (5, 6). In animals ingesting the iodoacetic acid-containing basal diet, all the aforementioned sulfur-containing amino acids support growth as well as does cystine. In addition, glutathione, which had previously been found to stimulate growth in animals given

<sup>6</sup> Acknowledgment is made to Dr. Robert Tennant, of the Department of Pathology, for making the histological examinations.

a cystine-low diet (19), and in animals stunted by naphthalene (20) or by methylcholanthrene feeding (5), also actively promoted growth in the present experiments.

In contrast to the above results, the unnatural isomer of cystine was unable to cause growth of rats stunted by means of iodoacetic acid. Two explanations may be suggested. It is possible that *d*-cystine may not be readily reduced to *d*-cysteine, a reaction which might be expected to be a prerequisite for the postulated combination of the sulfhydryl group with iodoacetic acid. The failure of the organism completely to oxidize *d*-cystine (21) indicates that metabolism of this isomer may proceed in part along lines differing from those believed to predominate in the case of the naturally occurring isomer of cystine. A second explanation is based on the hypothesis of Stekol (22), suggesting that cystine for detoxication is drawn from the tissues. The failure of *d*-cystine to stimulate growth in iodoacetic acid-inhibited animals would thus be related to the inability of the dextrorotatory isomer to enter into the tissue protein as a structural component.

The compounds *l*-phenyluraminocysteine, taurine, thioglycerol, and sodium sulfate were tested as sources of various forms of sulfur. The failure of these substances to promote growth demonstrates that the ability to stimulate growth in animals ingesting the iodoacetic acid-containing basal diet is not a general property of sulfate or sulfhydryl sulfur, but rather depends upon the specific chemical requirement for cystine or methionine or both or for compounds yielding one or both of these sulfur-containing amino acids in metabolism.

Laszt and Verzár (14) have reported that riboflavinphosphoric acid was capable of stimulating growth in rats stunted with iodoacetic acid; riboflavin was without effect under these conditions. In the studies reported here, the growth-promoting properties of these two compounds were compared. The effects on growth rate produced by the injection of either riboflavin or riboflavinphosphoric acid into animals ingesting the iodoacetic acid-containing basal diet were practically identical (0.05 and 0.13 gm. average daily weight decline, respectively). It is concluded that riboflavinphosphoric acid does not promote growth in animals ingesting iodoacetic acid under the experimental conditions employed. Laszt and Verzár (14) also claimed that the

administration of a cortical extract (eucortone) was effective in promoting growth in iodoacetic acid-fed animals. The latter investigators postulated that the cortex of the adrenal functions antagonistically to iodoacetic acid and that an excess of the adrenal cortical hormone allows the organism again to resume normal phosphorylation processes. In the present study, however, when cortin was injected in an amount sufficient to maintain a completely adrenalectomized animal in a healthy condition, no stimulus to growth was observed in rats stunted by iodoacetic acid. It may be pointed out that the level at which iodoacetic acid was fed in the present study was considerably higher (1 part of iodoacetic acid in 1000 parts of basal diet) than that employed by Laszt and Verzár (1 part of iodoacetic acid added to 5000 parts of a milk-grain diet). Moreover, the latter investigators' animals weighed only 50 gm. at the time that the halogen-containing acid was incorporated into the diet.

The data which have been obtained support the general hypothesis (5) that the inhibition of growth produced in young rats by a wide variety of toxic compounds is related to the demands placed upon the organism by detoxication mechanisms involving either of the sulfur-containing amino acids, cystine and methionine. The detoxication requirements of the organism for these amino acids apparently take precedence over the needs for growth, and on a relatively low protein diet, with a limited supply of cystine and methionine, a wide variety of compounds may then produce growth inhibition. The addition of either of these two sulfur-containing amino acids, or of compounds which may yield either cystine or methionine in the organism, makes available material for both detoxication mechanisms and for the synthesis of new tissue protein. Under these conditions, a prompt resumption in growth occurs, despite the continued presence in the diet of the toxic foreign compound. It is realized that convincing proof for this hypothesis includes the demonstration by isolation of a detoxication product, involving one of the sulfur-containing amino acids, from the urine of the experimental animal following the administration of the toxic substance. Evidence of this type has been obtained only after the administration of bromobenzene (22, 23), naphthalene (22, 24), and anthracene (25).

## SUMMARY

Growth inhibition has been produced in young rats by the addition of iodoacetic acid to a basal diet of relatively low protein content. The superimposition of *l*-cystine, *l*-cysteine hydrochloride, *dl*-methionine, or *dl*-homocystine on this iodoacetic acid-containing basal diet results in a prompt stimulation of growth with a resulting weight gain comparable to that observed in animals ingesting the basal diet alone. Subcutaneously injected *l*-cysteine hydrochloride or glutathione was also capable of stimulating growth in animals stunted with iodoacetic acid. On the other hand, the addition of *d*-cystine, taurine, anhydrous sodium sulfate, or *l*-phenyluraminocysteine to the basal diet containing the iodoacetic acid did not stimulate growth under the experimental conditions employed. Subcutaneously administered thioglycerol, riboflavin, riboflavinphosphoric acid, or cortical extract was also without effect on the inhibited growth rate. It is concluded that iodoacetic acid probably imposes a specific demand on the organism for the sulfur-containing amino acids, cystine or methionine, for detoxication purposes. Either of the latter compounds, or compounds yielding cystine or methionine in metabolism, may stimulate growth in the presence of iodoacetic acid by fulfilling the requirements for detoxication mechanisms and for the synthesis of tissue protein.

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# THE EFFECT OF DEPLETION OF EXTRACELLULAR ELECTROLYTES ON THE CHEMICAL COMPOSITION OF SKELETAL MUSCLE, LIVER, AND CARDIAC MUSCLE\*

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The work of Gamble, Ross, and Tisdall (1) showed that, since the concentration of total base in serum tended to be held relatively constant, the volumes of the intracellular and extracellular phases of body water must be closely related respectively to the amounts of potassium and sodium in the body. However, both in disease (2) and in experimental animals (3) large losses of extracellular electrolyte may not be accompanied by excretion of sufficient water to maintain electrolyte concentrations at normal levels. When serum electrolyte concentrations are abnormal, indirect methods indicate that osmotic equilibrium is attained largely by shifts of water between the two phases of body fluid. However, difficulties (4-6) encountered in applying these simple concepts show that, unmodified, they are only rough approximations. Studies of the reactions in individual tissues are apparently necessary to clarify the factors controlling the metabolism of water and electrolyte. The present paper gives the results of the analyses of liver, heart, and muscle of cats subjected to experimental losses of extracellular electrolyte.

Previous work has shown that increases in intracellular water of muscle accompany decrease in concentration of sodium in serum (7-10). However, it is now known that the proportion of potassium of muscle in relation to nitrogen and phosphorus may show large variations (7, 10-12) and it is now apparent that con-

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siderable and variable amounts of sodium occur in muscle cells (9, 11-13). The magnitude of the shifts of water which accompany changes in concentration of extracellular electrolyte is not known and the relation of these changes to alterations in the electrolyte pattern of the cell has not been systematically investigated. In connection with the present investigation, the analyses of the brain showed that losses of potassium apparently were the chief means of attaining osmotic equilibrium between intracellular and extracellular fluids, disturbed by loss of extracellular electrolyte (14). Data which show the simultaneous changes in tissue water and electrolyte are not available for the heart. Although deposition of glycogen has been shown to be accompanied by retention of water, potassium, and sodium in the liver (15), but little is known of the osmotic adjustments between the two phases of liver water and the variations in tissue electrolyte. Apparently the properties of cellular membranes cannot be regarded as limiting to shifts of water the response of cells to changes in the concentration of extracellular electrolyte.

### *Procedure*

The cats were given the usual laboratory diet for at least 1 week before the procedures to be described were carried out. Varying degrees of depletion of extracellular electrolyte were produced by the injection of 75 to 125 cc. of 5 per cent glucose solution intraperitoneally and subsequent removal of the fluid after 5 hours (3). The procedure was repeated the following day when more severe depletion was desired. In one group of animals so depleted, the fluid removed from the peritoneal cavity was replaced by an equal amount of 1 per cent solution of sodium sulfate. This procedure was evolved after it was found that when sodium sulfate was not given cats did not survive the double removal of extracellular electrolyte except in rare cases. All manipulations were carried out under nembutal anesthesia. The animals were killed 24 hours after the last depletion by severing the great vessels at the base of the heart.

The chemical methods are given in previous publications (7, 13).

Serum analyses are expressed per liter of serum in the case of potassium, and per liter of serum ultrafiltrate in the case of sodium and chloride. The concentration in the ultrafiltrate was calcu-

lated from the content of serum water and the Gibbs-Donnan factor of 0.96. For reasons given elsewhere (10) tissue analyses are expressed per 100 gm. of fat-free solids.

The statistical analysis was carried out according to the methods described by Dunn (16). Significant statistical correlations between the various factors were checked for rectilinearity by spot diagrams.

### *Results*

For simplicity in presentation the animals have been divided into four groups. Group I includes the normal animals, killed after a fast of 24 hours. Group II comprises the animals depleted of extracellular electrolyte. The animals in Group III were also depleted of varying amounts of extracellular electrolyte but in addition received sodium sulfate as described above. Group IV makes up all the animals included in the first three groups.

The essential points can best be brought out by statistical treatment of the data. These have been summarized in Table I, which gives the number of animals in each group, and the means and standard errors of the various components of the serum and tissues. The last column gives the concentrations of sodium plus potassium in the tissue water in mM per liter.

### *Changes in Serum*

The sera of the two groups depleted of extracellular electrolyte show certain differences. Those injected with sodium sulfate solution include the more severely depleted animals and consequently exhibit a greater average reduction in chloride. Sodium, however, is slightly higher. While the animals subjected to simple depletion have abnormally high concentrations of potassium, this is, as a rule, below the normal value in those injected with sodium sulfate. Presumably the oliguria and diminished glomerular filtration (17, 18) which develop in subjects with large deficits of extracellular electrolytes lead to retention of potassium when sodium sulfate solution is not given. However, the effect of sodium sulfate solution is apparently not attained solely by increasing the volume of glomerular filtration, since the non-protein nitrogen in Group III is definitely elevated ( $69 \pm 8$  mg. per cent)

TABLE I

Analyses of Serum, Muscle, Liver, and Heart of Normal Animals and of Animals Depleted of Extracellular Electrolytes

Group No.	No. of animals	Serum ultrafiltrate		Serum [K] <sub>s</sub>	Tissue, per 100 gm. fat-free solids							$\frac{(\text{Na}) + (\text{K})}{\text{H}_2\text{O}}$ = (B <sup>+</sup> ) <sub>t</sub>
					(H <sub>2</sub> O) <sub>t</sub>	(N) <sub>t</sub>	(Cl) <sub>t</sub>	(Na) <sub>t</sub>	(K) <sub>t</sub>	(P) <sub>t</sub>		
		[Cl] <sub>e</sub>	[Na] <sub>e</sub>									
Muscle												
I. Normal.....	34	mM per l. 132.6 ±0.6	mM per l. 155.8 ±0.6	mM per l. 5.7 ±0.1	ml. 345 ±3.2	gm. 15.4 ±0.03	mM 5.9 ±0.21	mM 8.0 ±0.21	mM 47.4 ±0.37	mM 33.5 ±0.27	mM per l. 160.2 ±1.1	
III. Depleted.....	19	102.8 ±2.9	134.2 ±1.7	7.1 ±0.5	373 ±4.4	15.1 ±0.06	5.0 ±0.16	6.9 ±0.36	49.5 ±0.42	33.7 ±0.19	152.3 ±2.6	
III. " (Na <sub>2</sub> SO <sub>4</sub> ).....	16	94.9 ±5.5	137.4 ±2.3	3.7 ±0.3	366 ±6.0	15.2 ±0.09	4.0 ±0.22	7.8 ±0.38	45.8 ±0.77	32.6 ±0.5	148.4 ±3.1	
IV. Total.....	69	115.7 ±2.5	145.6 ±1.4	5.8 ±0.3	358 ±2.9	15.3 ±0.03	5.2 ±0.16	7.7 ±0.17	47.6 ±0.32	33.4 ±0.17	155.3 ±1.3	
Liver												
I. Normal.....	13	130.2 ±0.9	152.6 ±1.7	4.8 ±0.2	270 ±3.4	12.0 ±0.28	11.2 ±0.33	11.2 ±0.23	30.4 ±0.5	36.2 ±0.6	153.5 ±1.7	
III. Depleted.....	19	104.0 ±3.1	134.6 ±1.8	7.1 ±0.5	316 ±6.1	12.4 ±0.25	9.7 ±0.35	11.1 ±0.42	34.3 ±0.6	37.9 ±0.9	143.3 ±1.8	
III. " (Na <sub>2</sub> SO <sub>4</sub> ).....	15	87.6 ±5.4	135.6 ±2.0	3.6 ±0.2	318 ±8.2	12.7 ±0.24	8.2 ±0.61	12.0 ±0.39	32.9 ±0.9	38.8 ±1.1	141.9 ±3.0	
IV. Total.....	47	106.0 ±3.0	139.9 ±1.7	5.3 ±0.4	304 ±4.8	12.4 ±0.15	9.6 ±0.3	11.4 ±0.22	32.8 ±0.5	37.7 ±0.6	145.7 ±1.4	

## Heart

I. Normal.....	24	132.2 ±0.15	154.1 ±1.1	4.7 ±0.13	410 ±5.5	14.1 ±0.18	19.1 ±0.4	24.4 ±0.9	40.1 ±0.7	34.0 ±0.6	158.5 ±1.9
II. Depleted.....	19	102.6 ±3.0	134.1 ±0.8	7.1 ±0.52	415 ±9.7	14.1 ±0.14	14.2 ±0.5	19.4 ±0.7	42.8 ±0.9	35.4 ±0.5	149.8 ±2.1
III. " (Na <sub>2</sub> SO <sub>4</sub> ).....	17	92.5 ±5.7	137.4 ±2.3	3.7 ±1.6	405 ±4.9	13.6 ±0.14	12.9 ±0.8	21.0 ±0.6	37.5 ±0.9	33.4 ±0.5	151.8 ±2.1
IV. Total.....	60	111.6 ±2.9	143.0 ±1.5	5.3 ±0.3	411 ±3.1	14.0 ±0.09	15.8 ±0.5	21.8 ±0.5	41.0 ±0.5	35.0 ±0.4	153.8 ±1.2

although not to as great an extent as in Group II ( $83 \pm 13$  mg. per cent). The finding of abnormally low values for potassium in the serum, muscle, and heart of Group III indicates that the excretion of potassium was promoted by the excretion of sodium sulfate.

### *Changes in Tissues*

*Muscle*—The concentration of tissue sodium and chloride decreases as the concentrations of these ions are diminished in the serum. The cats having a tendency to high concentrations of potassium in the serum (Group II) also show muscle potassium contents that are significantly higher than those in the control cats. Conversely, the group showing a tendency to low concentrations of serum potassium (Group III) has significantly low muscle potassium. Both groups of cats depleted of extracellular electrolyte show significant increases in tissue water, but no significant difference from each other in this respect. Groups II and III show a decrease in the concentration of sodium plus potassium in tissue water but in neither case is the reduction as great as the decrease of sodium in the serum. Nitrogen and phosphorus show no significant changes.

*Liver*—As in muscle, there is an increase in water and a decrease in chloride in the liver of the depleted animals. Moreover, in Group II there is a significant increase in potassium. The values for sodium show no change. There is an increase in nitrogen and phosphorus in the depleted animals of a proportionately equal magnitude. Although glycogen determinations were not carried out, presumably there was a decrease in the glycogen content of the livers of the depleted animals which were fasted from 24 to 48 hours longer than the controls. Since the values are expressed in terms of fat-free solids, the loss of glycogen would result in higher values for all the other constituents. This probably explains the changes in nitrogen and phosphorus. It should be noted, however, that the changes in water and potassium are too large to be explained on this basis. When the values are expressed with nitrogen or phosphorus as the unit of reference, the increases of water in Groups II and III and of potassium in Group II are equally significant. As in muscle, the decrease in the concentration of sodium plus potassium in liver water is significantly less than the decrease in serum sodium.

*Heart*—In the heart, there is a decrease in sodium and chloride proportional to the decrease of these ions in the serum. The increase in potassium in Group II and decrease in Group III, as noted in muscle, are also evident. Unlike muscle or liver, however, there is no significant change in tissue water. Nitrogen and phosphorus show relatively constant values. As in the case of

TABLE II

*Significant Correlation Coefficients between Various Constituents of Muscle, Liver, and Heart, and Ultrafiltrate of Serum*

Where no value appears, the correlation coefficient was not considered significant (less than twice the standard deviation of the coefficient).

Group No.*	(Cl) <sub>t</sub> [Cl] <sub>s</sub>	(Na) <sub>t</sub> [Na] <sub>s</sub>	(H <sub>2</sub> O) <sub>t</sub> [Na] <sub>s</sub>	(K) <sub>t</sub> [Na] <sub>s</sub>	(K) <sub>t</sub> (N) <sub>t</sub>	(P) <sub>t</sub> (N) <sub>t</sub>	(H <sub>2</sub> O) <sub>t</sub> (K) <sub>t</sub>	(P) <sub>t</sub> (K) <sub>t</sub>	(Na) <sub>t</sub> (K) <sub>t</sub>	(H <sub>2</sub> O) <sub>t</sub> (Na) <sub>t</sub>	(B <sup>+</sup> ) <sub>t</sub> [Na] <sub>s</sub>
Muscle											
I							0.76	0.73	0.62	0.71	
II		0.58	-0.57				0.47				0.82
III	0.69		-0.67								0.76
IV	0.55	0.51	-0.77				0.39	0.59			0.68
Liver											
I		0.70			0.82	0.86	0.73	0.75			
II	0.55		-0.61		0.58	0.88	0.80	0.64			0.73
III	0.78				0.67	0.74	0.57	0.68	0.72	0.71	
IV	0.78			-0.54	0.61	0.81	0.75	0.64			0.58
Heart											
I											
II	0.73	0.72								0.82	
III	0.88	0.58									
IV	0.83	0.64								0.55	

\* Group I normal, Group II depleted, Group III depleted and injected with Na<sub>2</sub>SO<sub>4</sub>, Group IV total.

muscle and liver the concentration of sodium plus potassium in the tissue water is decreased in the depleted animals, but to a lesser degree than is the sodium in the serum.

### *Correlation of Components*

The significant correlation coefficients between the various components of the tissues and serum in the different groups have

been summarized in Table II. Where no value appears, the respective correlation coefficient was not considered significant. The relationships between the various components will be described by means of regression equations which were determined from the data for the total group (Group IV) unless otherwise specified. In the subsequent discussion figures enclosed in parentheses represent tissue components and those enclosed in brackets, concentrations in the serum ultrafiltrate. All values are expressed as in Table I. The concentrations of sodium and chloride in the serum ultrafiltrate are understood to represent the concentrations of these ions in the extracellular fluid.

*Muscle.*  $(Cl)_t-[Cl]_e$ .—The relationship between muscle chloride and the concentration of chloride in the extracellular water is expressed by the following regression equation,  $(Cl)_t = 0.036[Cl]_e + 1.06 \pm 1.08$ ,<sup>1</sup> in which the standard error of the regression coefficient is 0.002, and of the intercept 0.13. The presence of a significant intercept in the equation indicates that within the range of chloride concentrations in extracellular fluid found in this study (60 to 140 mm per liter) a small fraction of the tissue chloride does not vary directly with changes in concentration of serum chloride. Analyses of several samples of muscle for red blood cells have indicated that erythrocytic chloride could account for only 0.05 mm per 100 gm. of fat-free solids. Manery, Danielson, and Hastings (19) have recently pointed out that connective tissue contains a relative excess of chloride over sodium. These authors estimate that in muscle about 1.0 mm of chloride per 100 gm. of fat-free solids may be associated with collagen rather than the ultrafiltrate of serum. This fraction of muscle chloride is similar in magnitude and may represent the non-diffusible chloride as described by the intercept in the regression equation.

$(Na)_t-[Na]_e$ .—The relationship between muscle sodium and the concentration of sodium in the extracellular fluid is expressed by the equation  $(Na)_t = 0.059[Na]_e - 0.9 \pm 1.8$ , in which the standard error of the regression coefficient is 0.012, and of the intercept 0.15. The fact that the regression coefficient is greater than that of the analogous chloride equation indicates that an apparently

<sup>1</sup> In this, and subsequent regression equations, this value represents the standard deviation of predictions calculated from the equation.

larger volume of fluid is required to contain diffusible muscle sodium than diffusible chloride at concentrations of a serum ultrafiltrate. Evidence from three laboratories (9, 11, 13) indicates that a considerable and variable amount of sodium in muscle is intracellular. The present data support this conclusion and also suggest that intracellular as well as extracellular sodium of muscle probably varies directly with the concentration of serum sodium.

$(H_2O)_i$ ,  $(K)_i$ ,  $(Na)_i$ ,  $[Na]_e$ —Muscle water varies directly with tissue sodium and potassium and inversely with the concentration of sodium in the serum. The correlation of tissue water with tissue sodium is shown only in the normal group, and the correlation with tissue potassium is highly significant only in this group. In the group as a whole, the variations in tissue water are mainly dependent on changes in the concentration of serum sodium, and, as is the case in the other tissues, almost entirely due to variations in intracellular water. The relationships may be expressed in the following regression equations.

$$\begin{aligned}(H_2O)_i &= -1.52[Na]_e + 579 \pm 15 \\ (H_2O)_i &= 3.52(K)_i + 190 \pm 22\end{aligned}$$

Since there was no correlation ( $r = 0.0$ ) between  $[Na]_e$  and  $(K)_i$ , these two equations may be combined directly as the multiple regression equation  $(H_2O)_i = 3.52(K)_i - 1.52[Na]_e + 411 \pm 14$ , in which the multiple correlation coefficient is 0.86.

When there is little variation in concentration of serum sodium, such as is found in normal animals (Group I), there is a high degree of correlation between tissue water and tissue sodium and potassium. For the normal cats, muscle water may be predicted by the following multiple regression equation  $(H_2O)_i = 4.5(K)_i + 5.8(Na)_i + 85 \pm 11$ , in which the multiple correlation coefficient is 0.82.

*Liver.*  $(Cl)_i$ ,  $[Cl]_e$ —The relationship between liver chloride and the concentration of chloride in the extracellular water is expressed by the following regression equation,  $(Cl)_i = 0.077[Cl]_e + 1.4 \pm 1.34$ , in which the standard error of the regression coefficient is 0.01, and of the intercept 0.2. As was the case in muscle, the presence of a significant intercept in the equation indicates that a small fraction of the liver chloride is presumably non-diffusible,



or at least does not react like a serum ultrafiltrate. That the distribution of liver chloride is not compatible with the hypothesis that all chloride occurs as an ultrafiltrate of the blood has been evident from the observations of other investigators who noted that the ratios of chloride to sodium in the liver and serum were such as to indicate a relative excess of chloride, assuming both ions were present as ultrafiltrates (11, 20).

The nature of the non-diffusible chloride represented by the intercept is not known. Analyses of livers in some cases for red blood cells indicated that erythrocytic chloride could account for approximately 0.05 to 0.07 mm per 100 gm. of fat-free solids. The possibility of the presence of connective tissue chloride, such as has been pointed out in muscle, was tested by separate analyses of the liver pulp and the more fibrous portions. These fractions were separated roughly by scraping. No significant differences in the chloride to serum ratios in the two fractions from the livers of two normal and six depleted cats were found. However, analyses of liver pulp and fibrous components do not exclude the presence of chloride in certain connective tissue cells not separated by the method employed.

$(Na)_i$ - $[Na]_e$ —The absence of significant correlation between liver sodium and the concentration of sodium in the extracellular water indicates that a considerable fraction of the tissue sodium is not present in the extracellular fluid, and not influenced by changes in serum sodium. It is not unlikely that the greater part of this fraction is intracellular but the evidence for this is only presumptive.

$(H_2O)_i$ - $(K)_i$ ,  $(Na)_i$ ,  $[Na]_e$ —As in muscle, liver water varies inversely with the concentration of sodium in the serum, and directly with liver potassium and sodium. The correlation of tissue water with potassium is highly significant in all groups, but, with tissue sodium, only in Group III. From the magnitude of the changes in concentration of serum sodium as compared to the changes in tissue potassium and sodium, it is evident that in the depleted animals the major factor responsible for the increase in tissue water is the decrease in serum sodium.

These relationships may be expressed by the following regression equations.

$$(H_2O)_i = -2.1[Na]_e + 595 \pm 23$$

$$(H_2O)_i = 7.7(K)_i + 51 \pm 21$$

The multiple regression equation involving both variables is the following,  $(H_2O)_t = 5.1(K)_t - 1.2[Na]_e + 302 \pm 18$ , in which the multiple correlation coefficient is 0.83.

$(N)_t$ ,  $(K)_t$ ,  $(P)_t$ —The presence of direct correlations between liver nitrogen, potassium, and phosphorus is presumably an expression of the presence of varying contents of glycogen in the livers. The point has been alluded to earlier in the discussion.

*Heart.*  $(Cl)_t$ ,  $[Cl]_e$ —The relationship between heart muscle chloride and the concentration of chloride in extracellular fluid is expressed by the following regression equation,  $(Cl)_t = 0.144[Cl]_e - 0.26 \pm 2.17$ , in which the standard error of the regression coefficient is 0.013, and of the intercept 0.28. Since there is no significant intercept, all tissue chloride may be considered diffusible and in equilibrium with the extracellular fluid.

$(Na)_t$ ,  $[Na]_e$ —The correlation between heart muscle sodium and the concentration of sodium in the extracellular fluid is expressed by the regression equation,  $(Na)_t = 0.22[Na]_e - 9.7 \pm 2.9$ , in which the standard error of the regression coefficient is 0.029. Obviously tissue sodium cannot be present entirely as an ultrafiltrate of serum and show the relation to extracellular sodium expressed in this equation. As in skeletal muscle, heart muscle sodium apparently occurs in at least two phases, as an ultrafiltrate of serum, and in the heart muscle cells. Assuming that the ratio,  $(Cl)_t:[Cl]_e$ , measures the volume of the fluid which reacts like an ultrafiltrate of serum, the average amount of intracellular sodium in Group I is 2.1, in Group II, 0.9, and in Group III, 1.8 m $\mu$  per 100 gm. of fat-free solids. This represents an approximate intracellular concentration of 8, 3, and 7 m $\mu$  per liter, respectively. As in the case of skeletal muscle, the intracellular sodium tends to be low when the tissue potassium is high, although there is scattering of the individual values. Moreover, there is no correlation between the concentrations of sodium in the extracellular and intracellular fluids. Although intracellular sodium may be in part controlled by the concentration of extracellular sodium and the concentration of intracellular potassium, the present data do not define the interrelationship adequately.

$(H_2O)_t$ ,  $(K)_t$ ,  $[Na]_e$ ,  $(Na)_t$ —Unlike the correlation in the case of skeletal muscle and liver, none could be demonstrated between tissue water and the concentration of sodium in the serum, or with tissue potassium. The correlation with tissue sodium, particu-

larly in the normal group, is apparently due to variations in the volume of extracellular fluid, since a significant correlation could also be demonstrated between tissue water and tissue chloride.

#### DISCUSSION

The data may be used to throw light on the nature of the osmotic adjustment occurring in intracellular water in response to decreases in the osmotic pressure of the extracellular fluids. Thermodynamic considerations indicate that approximate osmotic equilibrium must be maintained between intracellular and extracellular fluids. If univalent base exerts the preponderant osmotic effect in both fluid compartments, the concentration of univalent bases will approximately measure the osmotic pressure.

In the case of skeletal muscle, assuming no change in the sum of tissue sodium and potassium, the same change in concentration of univalent base in muscle water will be maintained when muscle water increases 2.3 ml. for each mm decrease in the concentration of serum sodium. However, in the regression equation expressing the indirect relationship between tissue water and the concentration of sodium in the extracellular fluid, the regression coefficient is  $1.52 \pm 0.15$ . In Table I it will be noted that in muscle the sum of sodium plus potassium remains fairly constant. The regression equation, therefore, indicates that tissue water changes only about two-thirds as much as the simple theory predicts. This is confirmed by the relationship between the concentration of sodium in the extracellular water and the concentration of sodium plus potassium in tissue water,  $(B^+)_t$ . The regression equation for this relationship is  $(B^+)_t = 0.62[Na]_e + 63.2 \pm 7.7$ , in which the standard error of the regression coefficient is 0.09. The change in concentration of base in tissue water is only about two-thirds as great as the simple theory demands and agrees with the equation predicting tissue water from the concentration of extracellular sodium.

The situation as regards the liver is somewhat different. Assuming no change in the tissue sodium and potassium, the same change in the concentration of univalent base in liver water would be maintained if the tissue water increased 1.9 ml. for each mm decrease in the concentration of extracellular sodium. Actually, the regression coefficient in the equation expressing the

relationship between liver water and serum sodium is  $2.1 \pm 0.3$ , a value not significantly different from that predicted by the simple theory. However, examination of Table I shows that the sum of sodium plus potassium in the tissue did not remain constant in the depleted animals. There occurred an increase in potassium which correlated with the decrease in concentration of serum sodium. Thus, the increase in tissue water proved to be only one-half as great as would have been necessary to maintain a change in the concentrations of univalent base in liver water equal to that of sodium in serum. This is brought out by the regression equation expressing the relationship between the concentration of sodium plus potassium in liver water,  $(B^+)_{\text{t}}$ , and the concentration of sodium in the extracellular fluid,  $(B^+)_{\text{e}} = 0.48[\text{Na}]_{\text{e}} + 78.7 \pm 7.4$ , in which the standard error of the regression coefficient is 0.10.

As regards the changes in the heart muscle, the data do not show relationships between tissue water and any of the various components that will explain a drop in the osmotic pressure within the cells comparable to that which occurs in the extracellular fluid. First, tissue water does not show an inverse relationship with serum sodium concentration. Second, tissue potassium shows but small fluctuations which could not account for alterations in the concentration of intracellular univalent base comparable to the variations in extracellular sodium. Third, the ratio  $(\text{Cl})_{\text{t}}:[\text{Cl}]_{\text{e}}$ , which approximately measures the volume of extracellular fluid, is relatively constant and indicates that significant variations in volume of intracellular water probably did not occur. Finally, there is no correlation between the concentration of sodium plus potassium in heart muscle water and the concentration of extracellular sodium (Table II).

It is, therefore, evident that as regards the three tissues studied, since changes in freely diffusible elements cannot account for the fact that the concentration of univalent base does not measure the osmotic pressure, changes in the osmotic pressure of some substances within the cells must accompany changes in the concentration of sodium in the serum. At present, the nature of the change or of the substances involved is not known. These findings show the difficulty of predicting the distribution of body water from balance experiments and suggest that shifts of water into cells

in response to decrease in concentration of extracellular sodium will be only about two-thirds that predicted by the assumption that the sole adjusting mechanism is transfer of water between the two phases of body water.

Amberson, Nash, Mulder, and Binns (21) reported that tissue chloride varies directly with serum chloride in a number of tissues, but that in certain tissues, notably brain and stomach, the intercept in the regression equation is so large that chloride cannot be entirely accounted for in the serum ultrafiltrate. Their experiments differed from ours, first, in that the shorter period of equilibration gave less assurance that even approximate equilibrium between blood and tissue fluids was attained and, second, in that chloride was displaced by sulfate rather than that both sodium and chloride were removed from the body. Although disagreeing with our results in the brain (14) their results in muscle, heart, and liver are comparable to ours. We agree with their view that each tissue must be examined from several points of view before it is decided what proportion of tissue chloride behaves as if it were present in an ultrafiltrate of serum.

The slope, or regression coefficient of the equation predicting tissue chloride from the concentration of chloride in serum, presumably measures the volume of water in which the diffusible chloride of the tissue exists at the concentration of an ultrafiltrate of serum. It follows that, in any tissue, this volume of fluid represents the maximum volume that may be assumed to react like an ultrafiltrate of plasma. The maximum volume will equal the true volume of extracellular water only if it can be shown that diffusible chloride exists only in the extracellular compartment. Conclusive evidence of the hypothesis that practically all diffusible chloride is extracellular is not available. Nevertheless the presumptive evidence is quite strong, since sucrose (22), thiocyanate, sulfate, and bromide (23) are all apparently dissolved in about the same volume of fluid. Our data indicate that in the calculation of the volume of extracellular fluid from the chloride concentrations in serum and tissues, 1 mm in the case of muscle and 1.4 mm in the case of liver should be subtracted from the total chloride of each 100 gm. of fat-free solids of the tissue.

Since the ratio of diffusible chloride in the tissues to the con-

centration of chloride in the extracellular fluid is constant, it may be assumed that the increases in muscle and liver water in the depleted animals are practically entirely confined to the intracellular compartment.

The data add to the evidence obtained in other types of experiments that tissue sodium cannot be entirely present as an ultrafiltrate of plasma. This is indicated by the differences between the ratios  $(\text{Na})_i : [\text{Na}]_e$  and  $(\text{Cl})_i : [\text{Cl}]_e$  in the individual tissues, as well as by the nature of the respective sodium regression equations. It would seem reasonable to assume that there is a considerable but variable quantity of sodium in the cells. However, the present data throw little light on the factors influencing the concentration of intracellular sodium. Heppel (11) has shown that in muscle, when cellular potassium decreases in response to a diet low in potassium, approximately equivalent amounts of sodium enter the cells. Similarly, in the skeletal and cardiac muscle of our depleted cats that received sodium sulfate solution, abnormally low tissue potassiums were in many instances associated with increases in tissue sodium that could not be related to increases in concentration of sodium in the serum, nor increases in volume of extracellular water. However, although it is suggested, the data do not show conclusively a reciprocal relationship between tissue sodium and potassium. This is indicated by the absence of significant correlation coefficients between these factors.

In Table I it will be noted that in the three tissues, an increase in tissue potassium is found in Group II in conjunction with an increase in serum potassium. Moreover, in skeletal and cardiac muscle, abnormally low values for tissue potassium in Group III are associated with abnormally low concentrations of serum potassium. Inspection of the individual values, however, shows that all levels of tissue potassium may occur with quite large variations in serum potassium, so that significant correlations between these two components could not be demonstrated in any of the groups. Thus, while common factors may influence in a similar fashion the concentration of potassium in the extracellular and intracellular fluids, there is no obligate relationship between the concentrations of potassium in the two compartments.

## SUMMARY

Cats were subjected to procedures leading to loss of extracellular electrolyte. Skeletal muscle, liver, cardiac muscle, and serum were analyzed for the principal inorganic anions and cations, water, nitrogen, and fat. Statistical analyses of the data indicated the following conclusions as regards these tissues.

Tissue chloride varies directly with the concentration of serum chloride in such a manner as to be compatible with the hypothesis that practically all tissue chloride is diffusible, and at the concentration of an ultrafiltrate of serum. A small fraction of the chloride in muscle and liver, however, does not vary directly with serum chloride.

Sodium in skeletal and cardiac muscle varies directly with the concentration of serum sodium but the evidence indicates that considerable and variable amounts occur in the intracellular phase as well as in the extracellular water. In the liver, there was no correlation between tissue sodium and the concentration of serum sodium.

The water of skeletal muscle and liver varies indirectly with the concentration of serum sodium. The increase of water in these tissues accompanying decrease in concentration of serum sodium is principally intracellular. In cardiac muscle the volume of tissue water was unaltered by depletion of extracellular electrolyte.

The relationship between the concentration of univalent base in tissue water and the concentration of sodium in serum indicates the following facts. In muscle and liver decrease in concentration of serum sodium is accompanied by a shift of water into the cells which is only sufficient to decrease the concentration of univalent base in the cells about one-half to two-thirds of that necessary for equal change in the concentration of univalent base in the two compartments. In the heart, decrease in the concentration of sodium in serum is unaccompanied by evidence of shift of water into the cells or of loss of univalent base from the cells. Apparently univalent base is only one of the factors determining the distribution of water between the intracellular and extracellular phases.

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## THE INFLUENCE OF THE TYPE OF CARBOHYDRATE INGESTED UPON CITRIC ACID PRODUCTION\*

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The "citric acid cycle" hypothesis of carbohydrate oxidation has stimulated an interest in the general relationship of citric acid to carbohydrate metabolism. Krebs and Johnson (1937, *a*) and Krebs (1937) have presented evidence which they believe demonstrates that a catalytic system composed of certain dicarboxylic acids and citric acid "outlines the principal pathway of the oxidative breakdown of carbohydrate in muscle tissue." According to their scheme, an increased rate of oxidation of carbohydrate would result in the elaboration of more citric acid. A precursor of the citric acid thus formed might be any other member of the cycle. Krebs and Johnson (1937, *b*) showed that succinic acid may be synthesized in small amounts by animal tissue *in vitro* if pyruvic acid is available. On the basis of this observation these authors infer that the origin of citric acid is explained.

Although Greenwald (1914) showed that citric acid is practically quantitatively converted to glucose in the human diabetic and in phlorhizinized dogs, definite evidence that the reverse reaction occurs in animals is lacking. The ingestion of glucose has been reported to have no effect in human beings either on the excretion of citric acid (Kuyper and Mattill, 1933) or on its concentration in the blood (Lennér, 1934). Sherman, Mendel, and Smith (1936) were unable to obtain convincing evidence that

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sucrose gives rise to citric acid; when dogs fed a citrate-low diet were given an increased amount of sucrose, no significant change in the urinary output of citric acid was noted. However, when sodium bicarbonate was administered, there was an indication that the resultant increased excretion of citric acid was further augmented by a carbohydrate-rich diet as contrasted with a protein-rich diet. More recently Smith and Meyer (1939) observed that rats excrete considerably more citric acid when maintained on diets rich in dextrin or fat than when protein (lactalbumin) provides a larger proportion of the dietary energy. They interpreted this observation to mean that the citric acid

TABLE I  
*Composition of Diets*

Each rat received a solution containing 80 mg. of liver extract, Lilly, No. 343, and 200 mg. of ryzamin-B concentrate per day (1.032 mg. of citric acid per 3 days).

	"Hexose"*	Sucrose	Dextrin and starch
Lactalbumin.....	14.0	14.0	14.0
Carbohydrate*.....	65.1	61.8	58.6
Crisco.....	20.0	20.0	20.0
Cod liver oil.....	5.0	5.0	5.0
Salt mixture†.....	2.4	2.4	2.4
	106.5	103.2	100.0

\* Glucose, fructose, or galactose.

† Hubbell, Mendel, and Wakeman (1937).

normally excreted is derived from metabolic products of fats and carbohydrates.

A question naturally arises as to whether the differences in results noted in the foregoing two investigations are due to differences in species or to the nature of the carbohydrates administered. The data presented in the present paper are derived from a study of the effects of feeding, to rats, diets rich in various carbohydrates.

#### EXPERIMENTAL

The experiments were conducted in the same manner as those described previously by Smith and Meyer (1939). The composi-

tion of the rations is given in Table I. The dextrin diet was the one used earlier (Smith and Meyer, 1939), while in the case of the glucose, fructose, galactose, and sucrose diets, adjustments were made in the quantity of the respective carbohydrate in order to provide an "effective" level equivalent to dextrin. Starch was used in the same amount as dextrin.

Groups of four rats were maintained on each of the diets for 21 days. Food consumption and weight changes were noted every 3 days. Urine for citric acid analyses was also collected at 3 day intervals. The citric acid was determined by the method of Pucher, Sherman, and Vickery (1936), the final measurement being

TABLE II

*Citric Acid Production of Rats on Various Carbohydrate Diets*

Each number represents the average citric acid production for one rat for seven 3 day periods.

Glucose	Fructose	Galactose	Sucrose	Starch	Dextrin
mg.	mg.	mg.	mg.	mg.	mg.
15.2	14.4	17.8	7.4	16.3	23.6
19.9	15.3	7.1	20.7	24.8	24.1
8.9	11.2	9.1	58.6	19.1	22.7
3.3	4.7	10.7	15.8	40.2	32.6
11.8	11.4	11.2	20.6	25.1	25.7

made with a photometer having a color filter with maximum transmission at 4250 Å.

As in earlier work, considerable variation in the citric acid production within the group on each diet was noted. However, fluctuations in the periodic quantity produced by any one rat were within rather narrow limits, so that a distinct individuality was discernible. Reference may be made to Table II in a previous paper (Smith and Meyer, 1939) for similar examples of this characteristic; only the average values for each rat are presented here and these data are recorded in Table II. The values presented represent the urinary excretion minus the quantities of citric acid ingested in the diets. The intakes of citric acid were negligible, with a maximum difference of 0.2 mg. per day due to the amounts present in the various diets.

While there was considerable individual variation on any one diet during the entire 21 days, a striking constancy prevailed in the average citric acid production of rats in the hexose groups. Contrasted with an average production of 11 mg. per 3 days on either the diet high in glucose, fructose, or galactose are the values of 25 mg. for the dextrin and starch groups. The rats

TABLE III

*Citric Acid Production of Rats on Diet High in Dextrin Compared with Diets High in Other Carbohydrates*

Each number represents the average citric acid production for one rat for five consecutive 2 day periods.

Diet				
Glucose	Fructose	Galactose	Sucrose	Starch
mg.	mg.	mg.	mg.	mg.
9.0	9.7	13.5	21.3	24.8
8.9	6.8	11.8	17.0	31.2
16.1	19.3	4.0	15.5	38.1
12.4	4.8	5.1	15.5	18.5
11.6	10.1	8.6	17.3	28.1
Dextrin*				
22.3	21.5	64.7	32.0	13.2
8.8	19.0	54.8	17.4	22.4
29.3	39.0	60.8	27.6	33.8
16.1	15.0	53.9	23.0	21.9 <sup>2</sup>
19.1	23.6	58.5	25.0	22.8

\* The contrasting values for an individual rat occupy the same relative positions in the upper and lower parts of the table.

maintained on the sucrose-rich diet produced an intermediate average quantity of 20.6 mg. of citric acid in 3 days.

Although not a single animal in the three hexose groups excreted as much citric acid as the *average* excretion in the other groups, in some cases considerable individual overlapping did occur. For instance, one rat which received the glucose diet averaged 19.9 mg., while the value for one animal given sucrose was only 7.4 mg. Likewise, another rat on the sucrose diet averaged 58.6 mg. of citric acid which was considerably greater

than that for any of the animals on the dextrin or starch diets. Owing to these deviations from the group averages, it might be concluded that one group excreted a greater quantity of citric acid than another merely because of a fortuitous selection of its members. Consequently, the influence of different carbohydrate diets upon the same animal was studied. It was believed that in this manner individual variability might be circumvented; if an animal excreted more than its experimental mates on one particular carbohydrate diet, it would continue to do so on another. The glucose, fructose, galactose, sucrose, and starch diets were given to groups of four rats each for 10 days and then all were

TABLE IV  
*Comparison of Citric Acid Excretion with "Effective"  
Carbohydrate Consumption\**

Diet	1st series		2nd series	
	Carbohydrate consumed	Citric acid excreted	Carbohydrate consumed	Citric acid excreted
	gm.	mg.	gm.	mg.
Glucose.....	6.66	3.9	7.58	5.8
Fructose.....	6.48	3.8	5.56	5.0
Galactose.....	7.15	3.7	6.66	4.3
Sucrose.....	8.01	6.8	6.05	8.7
Dextrin.....	6.98	8.9		
Starch.....	7.55	8.4	7.10	14.0

\* "Effective" carbohydrate consumption per rat per day is calculated on the basis that all carbohydrates are converted to hexoses.

replaced by the dextrin diet for another 10 days. The effects of these regimens on the excretion of citric acid are shown by the data in Table III. During the fore period the animals comprising the sucrose and starch groups again excreted more citric acid than the rats fed hexose diets. Concomitantly with the change to the dextrin diet, immediate adjustments in the production of citric acid were noted; there was a prompt increase in the case of the hexose and sucrose groups with a slight decrease in the starch group. The most striking response was manifested by the animals which were changed from the galactose to the dextrin diet, a 7-fold increase resulting from this substitution.

An accurate record of food consumption was kept. Table IV contains the daily average individual carbohydrate consumption, expressed as hexose, of the rats on the various diets. These values are compared with the corresponding quantities of citric acid excreted. It is obvious that the differences in citric acid values are not due to a quantitative difference in consumption of the various carbohydrates.

Why sucrose, dextrin, and starch, carbohydrates which are hydrolyzed to glucose before absorption, are more effective promoters of citric acid excretion than is glucose is not clear. It was thought pertinent to determine the relative excretion of citric acid of rats consuming diets containing equivalent quantities of glucose, invert sugar, and sucrose. Four rats were placed upon each of such diets, but it was not possible to distinguish sufficiently between the quantities of citric acid eliminated to reach a definite conclusion. Accordingly, six other animals were given the diet high in glucose for 10 days, and then transferred to the sucrose-rich diet with a view to subsequent transfer to one containing an equivalent of invert sugar. The excretion of citric acid on the two diets did not consistently differ sufficiently to offer a basis for comparison, so that the invert sugar regimen was not instituted. All the rats were then transferred to a diet high in dextrin and subsequently to the glucose ration. When this was done, the excretion of citric acid dropped from a grand average of 29.3 mg. for four 2 day periods on the dextrin to 17.2 mg. on the glucose, showing again the same relative effectiveness of these two carbohydrates as observed with the first series of animals.

It was thought that possibly intestinal bacteria might be responsible for the differences that were observed, for it is known that the polysaccharides are more conducive to bacterial growth than are simpler sugars. To our knowledge there are no reports in the literature on the synthesis of citric acid by bacteria. To test this possibility, two groups of six rats each were fed diets which give maximum differences in urinary citric acid excretion. One group was fed the "standard" diet previously used by Smith and Meyer (1939), while the other group received the galactose diet for 6 days and was then transferred to the one high in dextrin. Although the second group had been conditioned for only this

brief period on the galactose diet, a 4-fold increase in urinary citric acid was observed when the transfer was made. Feces were also collected and analyzed for citric acid. After 6 days on the standard diet, the first group was sacrificed and their intestinal contents and intestines were analyzed. The second group was similarly treated after it had been on the dextrin diet for 6 days. The average fecal excretion for two 3 day periods for the first group was 1.8 mg. and 3.0 mg. and for the second group 2.2 and 2.8 mg. The average quantities of citric acid found in the intestines and their contents were 1.1 mg. for both groups.

This experiment did not exclude the possibility that rapid absorption might mask a differential synthesis by the bacteria.

TABLE V

*Effect of Intestinal Bacteria upon Small Quantities of Citric Acid*

Carbohydrate	Quantity of carbohydrate	Citric acid	
		Control	Experimental
	gm.	mg.	mg.
Galactose	0.20	0.42	0.29
	0.20	0.42	0.29
Sucrose	0.19	0.42	0.32
	0.19	0.42	0.32
Dextrin	0.18	0.44	0.24
	0.18	0.44	0.32

To test further the possibility of bacterial synthesis, their effect upon different carbohydrates was determined *in vitro*. 8 gm. of intestinal contents from a rat were diluted to 100 cc. with normal saline. 5 cc. of this suspension were used in each determination indicated in Table V. To each tube were also added 40 cc. of a solution containing 0.32 gm. of desiccated beef broth and 0.32 gm. of sodium chloride, and the quantity of the carbohydrate indicated in Table V. The controls which were made for each carbohydrate were immediately heated with 5 cc. of 50 per cent sulfuric acid. The others were kept in an incubator at 37° for 66 hours and then analyzed for citric acid. The results indicate that no citric acid was produced; rather, in each case, there was a slight destruction of this compound.



## DISCUSSION

It is apparent from the present work and that previously presented (Smith and Meyer, 1939) that the quantity of citric acid produced by an animal is governed to a considerable extent by the type of diet ingested. That this response is an intrinsic property of the dietary constituents rather than a reflection of the well known acid-alkali influence on citric acid excretion is now especially evident. Certain carbohydrates change the pH of the intestine through their influence on the type of bacterial flora in the gut and thus would favor the absorption of acid. It is, however, the polysaccharides which favor the acidophilic intestinal bacteria, and hence, from the known influence of acid-base balance on citric acid synthesis, should effect a *decreased* elimination of citric acid. This is not in accord with the results obtained.

Although it was found that rats which ate a diet high in dextrin did not excrete more citric acid in the feces than others on the standard diet (while they were excreting considerably more in the urine) and that no more was present in their intestines immediately after death, this alone does not exclude a synthesis by bacteria in the gut. However, the fact that intestinal bacteria destroyed small quantities of citric acid *in vitro* renders synthesis by these organisms unlikely.

Bacteria do, of course, synthesize other organic acids, and some of these *might be* precursors of citric acid. Whether the quantities of such organic acids produced by bacteria are large enough to cause an increase in the urinary citric acid is questionable, for when large quantities of citric acid itself are given to various species of animals, it is virtually completely oxidized, rarely more than 2 per cent appearing in the urine. On this basis, the intestinal flora of the rats which had been changed from a galactose-rich to a dextrin-rich diet would have had to synthesize an *equivalent* of over 1.4 gm. of citric acid per day in order to account for the 29 mg. in the urine of this group. Further evidence against bacterial synthesis as the responsible factor in the increase in urinary citric acid is provided by the data of Kuether, Meyer, and Smith (1940) which show that the oral administration of free citric acid to the rat actually results in a diminution of its excretion in the urine.

At present no satisfactory explanation can be given for the stimulating effect of carbohydrates on the endogenous production of citric acid, much less for the difference between the monosaccharides and the polysaccharides in this respect. The exceptional production of citric acid by the galactose-fed animals when transferred to a high dextrin diet recalls the uncommon behavior of this sugar as regards ketolysis and the formation of glycogen (see Deuel (1936)).

#### SUMMARY

It has been observed that when carbohydrate comprises 65 per cent of a ration, expressed as glucose, the individuality of the carbohydrate has an influence on the amount of urinary citric acid excreted by rats. Whereas the excretion of citric acid was practically the same when the diets contained this quantity of glucose, fructose, or galactose, it was more than twice as much when the carbohydrate was dextrin or starch.

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## THE *d*-AMINO ACID OXIDASE CONTENT OF RAT TISSUES IN RIBOFLAVIN DEFICIENCY\*

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The elucidation of the structure of a number of the coenzymes which are involved in many of the oxidative processes of the cell has produced the significant observation that at least three vitamins are essential constituents of such coenzymes. Thus, thiamine and nicotinic acid amide have been shown to be components of cocarboxylase and the pyridine nucleotides respectively (1-3). The situation is more complex with respect to riboflavin. Until quite recently riboflavin, as riboflavin phosphate, was recognized only as a constituent of Warburg's "yellow ferment." Within the past 2 years the rôle of riboflavin in enzymatic processes has assumed greater significance through the knowledge that there exists a whole group of enzymes with prosthetic groups containing riboflavin, usually as riboflavin-adenine dinucleotide. In this group are included "new" yellow enzyme, Warburg and Christian (4); *d*-amino acid oxidase, Warburg and Christian (5); yeast flavoprotein, Haas (6); milk flavoproteins with diaphorase and xanthine oxidase activities, Corran and Green (7), Ball (8); heart muscle flavoproteins with diaphorase activity, Straub (9), Adler *et al.* (10); liver flavoproteins with xanthine oxidase activity, Corran *et al.* (11) and Subrahmanyam *et al.* (12); yeast fumarate hydratase, Fischer *et al.* (13); and a constituent of the bacterial pyruvate oxidase system, Lipmann (14).

The relationship of the above observations to the physiological

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rôle of these vitamins becomes apparent. It is reasonable to assume that these vitamins function as precursors of their respective coenzymes and that pathological manifestations in the given vitamin deficiencies are due in part to a disturbed metabolism resulting from a deficiency of the essential coenzyme. Such coenzyme deficiencies have been observed in thiamine (15), nicotinic acid (16-18), and riboflavin avitaminoses (19).

We have previously reported in a preliminary communication (20) that a riboflavin deficiency results in a lowered *d*-amino acid oxidase content of rat liver and kidney. Ochoa and Rossiter (19) have demonstrated similar decreases in the total riboflavin-adenine dinucleotide content of rat heart and liver in riboflavin avitaminosis. In this paper we shall present a more detailed account of our work with particular reference to the effect of other members of the vitamin B complex upon the *in vivo* synthesis of *d*-amino acid oxidase.

### Methods

*Treatment of Animals*—Two basal rations were employed to produce deficiencies in various members of the vitamin B complex. The less synthetic Ration K-12 is low in riboflavin, factor W, vitamin B<sub>6</sub>, and possibly pantothenic acid (21). The highly synthetic basal Ration J-30 is devoid of all known members of the vitamin B complex required by the rat except those already added (Table I).

Albino rats of both sexes were placed on experiment at 21 days of age. The groups receiving basal Ration K-12 were depleted for 6 to 8 weeks, while those receiving Ration J-30 were depleted for 3 weeks. The animals were fed *ad libitum* with the exception of one group receiving Ration K-12 plus riboflavin plus factor W concentrate whose food intake was restricted to that of a group receiving only basal Ration K-12. At the end of the respective depletion periods, growth had ceased in both groups. Symptoms of alopecia were observed in a number of rats receiving basal Ration K-12. No external symptoms were evidenced in the group fed Ration J-30. The rats were then placed in individual, wire-bottomed cages and, in addition to their basal ration, were supplemented with various members of the vitamin B complex for 2 to 4 weeks. The grouping of the rats according to the supplements

received is given in Table II. The large majority of the rats consumed their supplements immediately although in a few cases it became necessary to withhold their water for a short period of time. The daily supplements consisted of (1) synthetic riboflavin, 40 micrograms, (2) synthetic vitamin B<sub>6</sub>,<sup>1</sup> 40 micrograms, (3) muscle adenylic acid,<sup>2</sup> 100 micrograms, (4) a factor W concentrate (23)  $\approx$  0.4 gm. of liver extract.<sup>3</sup> This concentrate was free of vitamin B<sub>6</sub> and riboflavin but furnished adequate amounts

TABLE I  
*Composition of Basal Rations K-12 and J-30*

Components	Ration K-12	Ration J-30*
White corn, gm.....	12.5	
Dextrin, gm.....	58	
Sucrose, " .....		76
Labco casein, gm.....	18.5	18
Salts 3 (22), " .....	4.0	4
Butter fat (washed), gm. ....	5.0	
Corn oil, gm.....		2
Cod liver oil, gm.....	2.0	
Thiamine hydrochloride, $\gamma$ .....	200	300
Nicotinic acid, mg.....		5
Choline hydrochloride, mg. ....		5

\* 2 drops of haliver oil were fed weekly to each rat.

of factor W and pantothenic acid at the levels fed. The animals were weighed weekly.

*Determination of d-Amino Acid Oxidase*—The kidney and liver were removed from a decapitated rat and freed from any adherent blood with moistened filter paper. A portion of the tissue was placed on solid carbon dioxide and reserved for the riboflavin analysis. Another portion was weighed into a tared homogenizer tube and immediately homogenized (24) with 5 volumes of dis-

<sup>1</sup> We are indebted to Merck and Company, Inc., Rahway, New Jersey, for generous supplies of thiamine, nicotinic acid, and vitamin B<sub>6</sub>.

<sup>2</sup> The muscle adenylic acid was purchased from the Laokoon Company, Lwow, Poland.

<sup>3</sup> We are indebted to Dr. David Klein, The Wilson Laboratories, Chicago, for the liver extract used in the preparation of the factor W concentrate.

tilled water. 1 cc. of the liver and 0.5 cc. of the kidney suspensions were used for the respective enzyme determinations. Not more than 20 minutes elapsed between the death of the animal and the beginning of equilibration. The rate of oxygen uptake of these tissue suspensions in the presence of *dl*-alanine was taken as a measure of the *d*-amino acid oxidase content of the tissue. Only *d*-alanine is oxidatively deaminized under these conditions (25). The oxygen uptake was measured in the Barcroft differential respirometer with potassium hydroxide in the inner well (26). The reaction was carried out with air as the gaseous phase. 3 cc. of water were placed in the left-hand flask, while the right-hand flask contained the following constituents: (1) 1.5 cc. of Kreb's phosphate buffer of pH 7.4 (27), (2) 0.5 cc. of 0.3 M *dl*-alanine, when required, and (3) the requisite amount of tissue. The contents were diluted to 3 cc. with distilled water. The neutralized substrate was prepared daily. The determinations were run at 37°. A blank oxygen uptake (in the absence of *dl*-alanine) was always determined for each tissue and the determinations in the presence of substrate were run in duplicate. The duplicates were consistently checked within 10 per cent. The contents of the flask were equilibrated for 10 minutes, the stop-cocks closed, and readings taken every 10 minutes thereafter for 1 hour. The rate of oxygen uptake remained constant for 20 minutes and then gradually decreased. The oxygen uptake in c.mm. per hour is expressed as the 20 minute reading (minus the 20 minute blank reading)  $\times 3$ . The blank readings did not vary significantly with the various groups.

The effect of pyruvate was studied and it was found that neither liver nor kidney suspensions could utilize amounts of pyruvate equivalent to that produced during the oxidative deamination of *d*-alanine under our experimental conditions. Pyruvate in the same amounts did not inhibit the reaction.

*Determination of Riboflavin Content*—The tissues were stored in the frozen state. There was no change in the riboflavin content of tissues when stored under these conditions for 3 months. The tissue was homogenized, diluted 1:500 with distilled water, and autoclaved for 15 minutes at 120°. Riboflavin was determined in this extract according to the bacteriological method of Snell and Strong (28). Feeney and Strong (29) have demonstrated

that this method measures not only free riboflavin but also the combined forms.

There was no variation in the moisture content of tissues taken from rats in the various groups.

#### EXPERIMENTAL

The data obtained are presented in Table II.

*Observations on Basal Ration K-12*—Our first experiments were designed to determine what relationship, if any, existed between the riboflavin intake of a rat and the *d*-amino acid oxidase content of its tissues. Such a relationship was postulated after it became known that the prosthetic group of this enzyme was riboflavin-adenine dinucleotide. It was also of interest to determine what effect other members of the vitamin B complex might have upon the synthesis of this enzyme. The latter purpose assumed more importance after it became evident that supplementation of the basal ration with riboflavin alone, although restoring the riboflavin content of the liver to its normal value, only partially restored its *d*-amino acid oxidase content. As given in Table II, various combinations of different members of the vitamin B complex were fed. Analysis of the mean values led to the following conclusions. The liver of rats fed basal Ration K-12 contained significantly lower amounts of *d*-amino acid oxidase. A smaller but significant lowering was also observed in the kidney. There was also a decrease in the riboflavin content, particularly of the liver. Supplementation of basal Ration K-12 with either a factor W concentrate or riboflavin alone resulted in an increase in the *d*-amino acid oxidase content of both the liver and kidney. Complete restoration of the enzyme content of the liver, when riboflavin was the only supplement, was not accomplished although the riboflavin content was restored to normal. Supplementation with both riboflavin and vitamin B<sub>6</sub> or riboflavin and factor W concentrate increased the *d*-amino acid oxidase content of both liver and kidney to their normal values. Restriction of the food intake of a group of rats receiving Ration K-12 plus riboflavin plus factor W concentrate to that of a group of rats receiving Ration K-12 alone prevented growth but allowed complete restoration of the *d*-amino acid oxidase content of both liver and kidney.



TABLE II  
Summary of Data\*

Supplements	Oxygen uptake†		Riboflavin content of tissues (fresh weight)		Weight‡ gain	No. of rats in each group
	Liver	Kidney	Liver	Kidney		
	c.mm. per hr.	c.mm. per hr.	γ per gm.	γ per gm.	gm. per day	
<b>Series on Ration K-12</b>						
None.....	20	340	23	37	0.0	6
Factor W concentrate.....	82	537	30	40	0.25	6
“ “ “ + vitamin B <sub>6</sub> .....	47	441	27	37	0.10	2
Riboflavin.....	82	480	40	46	2.1	7
“ + vitamin B <sub>6</sub> .....	144	577	42	46	2.5	3
“ + factor W concentrate.....	127	507	40	47	3.0	10
Riboflavin + factor W concentrate (restricted).....	156	542	42	45	0.69	5
Riboflavin + factor W concentrate + vitamin B <sub>6</sub> .....	117	540	48	46	2.9	4
Stock ration.....	129	524	54	51		6
<b>Series on Ration J-30</b>						
Muscle adenylic acid.....	50	414	36	44	0.0	6
Riboflavin + adenylic acid....	129	426	48	50	0.10	11
“ + “ “ + factor W concentrate.....	169	669	46	49	0.89	4
Riboflavin + adenylic acid + factor W concentrate + vitamin B <sub>6</sub> .....	87	550	51	59	3.2	4
Riboflavin.....	179	583	51	48	0.0	6
“ + vitamin B <sub>6</sub> .....	113	403	47	52	0.45	6
Vitamin B <sub>6</sub> .....	45	451	34	40	0.0	4

\* The mean values for each group are given.

† Oxygen uptake values are expressed as the 20 minute reading (minus the 20 minute no substrate reading)  $\times 3$ . 1 cc. of a 1:6 liver suspension and 0.5 cc. of a 1:6 kidney suspension were used.

‡ Weight gains represent gains during the period of supplementation. Sustained growth is obtained only in the presence of factor W, vitamin B<sub>6</sub>, and riboflavin.

*Observations on Basal Ration J-30*—This ration was adopted because it was thought that further information concerning the relationship of other members of the vitamin B complex to the

synthesis of *d*-amino acid oxidase could be obtained with the use of a more synthetic basal ration. An analysis of these data led to the following conclusions.

In the absence of riboflavin there was a significantly lower amount of *d*-amino acid oxidase in liver. No significant decreases were observed in the kidney. It is important to note that the riboflavin content of the liver was not decreased to the same extent as that of the corresponding group of rats fed basal Ration K-12. This may be due to the fact that the animals on Ration J-30 were not depleted for as long a period of time. This difference in riboflavin content was paralleled by the observation that the enzyme content of the riboflavin-deficient rats from the J-30 group was not as low as that on basal Ration K-12. The addition of riboflavin to this ration (No. J-30) completely restored the normal content of *d*-amino acid oxidase. Further additions of (1) adenylic acid, (2) adenylic acid plus factor W concentrate, (3) adenylic acid plus factor W concentrate plus vitamin B<sub>6</sub>, and (4) vitamin B<sub>6</sub> alone had no supplementary effects.

Various differences between the K-12 and J-30 groups were observed. The addition of riboflavin to basal Ration K-12 resulted in a temporary growth response accompanied by only a partial increase in the *d*-amino acid oxidase content of liver. No growth response was obtained upon the addition of riboflavin to basal Ration J-30 although complete restoration of the enzyme content was effected. There appears to be a general correlation between the amount of growth and the degree of *d*-amino acid oxidase synthesis. Where rapid growth takes place, as in the group fed Ration K-12 plus riboflavin, other factors necessary for such a synthesis of the enzyme may become limiting. Such deficiencies are corrected by the addition of either vitamin B<sub>6</sub> or factor W concentrate to Ration K-12. It is interesting to note that a very uniform synthesis was observed in the animals receiving Ration K-12 plus riboflavin plus factor W whose growth had been prevented by restricting the food intake. A similar explanation would account for the complete synthesis that took place in the group fed Ration J-30 plus riboflavin in the absence of other factors and in the complete absence of growth. Evidence that factors other than those already mentioned may be required for maximum enzyme synthesis on Ration J-30 when growth is

optimum may be derived from the failure to effect complete synthesis in the group fed Ration J-30 plus riboflavin plus factor W concentrate plus vitamin B<sub>6</sub> plus adenylic acid. Optimum growth was obtained in this group.

#### DISCUSSION

It should be borne in mind that the enzyme under consideration is one which has as its specific substrate a type of amino acid, *i.e.* the *d* form, which is generally considered foreign to the animal body. The significance of such an enzyme is not entirely clear at the present time. This must be considered before any attempts are made to correlate a *d*-amino acid oxidase deficiency with any specific syndrome. The study of the *l*-amino acid oxidase would perhaps represent a truer physiological approach to this problem. In this connection it is of interest that Russell and Wilhelmi (30) have observed that kidney slices from adrenalectomized rats are not able to utilize *l*(+)-glutamic acid and *dl*-alanine as well as normal kidney slices. This type of evidence suggests a deficiency of both the *d*- and *l*-amino acid oxidase in the kidney of the adrenalectomized rat. Lyman and Barron (31) have shown that kidney slices from rats made nephritic by the ingestion of diethylene glycol possessed a decreased ability to oxidize *dl*-alanine and *l*-aspartic acid. Thus it is apparent that other conditions besides a riboflavin deficiency may affect the *d*- and *l*-amino acid oxidase content of tissue.

The lowered riboflavin content of tissues from rats fed a riboflavin-low ration is in general agreement with previous observations (32-36). The values for the total riboflavin-adenine dinucleotide content of normal rat liver and kidney given by Ochoa and Rossiter (19) check well with our figures for the riboflavin content of these tissues if it is assumed that all of the riboflavin is present as the dinucleotide. The decrease in the total riboflavin-adenine dinucleotide content of liver observed by these workers is of the same order as our observations on the riboflavin decrease in this tissue.

Our experiments do not enable us to determine which component of the *d*-amino acid oxidase enzyme is affected in a riboflavin deficiency. Decreases in the concentrations of either the riboflavin-adenine dinucleotide or in the protein component, or

perhaps in both, would result in a lowered *d*-amino acid oxidase activity. Ochoa and Rossiter (19) have demonstrated that there is a lowered total riboflavin-adenine dinucleotide content of the heart and liver in a riboflavin deficiency. An analysis of the specific protein of the *d*-amino acid oxidase, such as that carried out by Klein (37), would determine whether the protein were also affected. The possibility of such a change in the protein component is indicated by the work of Klein (37) who found that the feeding of thyroid resulted in an increase in the concentration of the protein component of the *d*-amino acid oxidase present in liver. A correlation between metabolic activity in the animal and the concentration of a protein constituent of an enzyme was clearly indicated. It is interesting to note that the metabolic changes did not affect the *d*-amino acid oxidase content of the kidney. This is in agreement with our findings that the changes in the liver were considerably more significant than those in the kidney. Lyman and Barron (31) are of the opinion that the ingestion of diethylene glycol affects the activating protein of a series of oxidizing enzymes in rat kidney.

It is apparent that the riboflavin-adenine dinucleotide coenzyme which is combined with the specific protein to form the *d*-amino acid oxidase represents only a portion of the total riboflavin-adenine dinucleotide content of tissue. The number of other enzymes which contain the same dinucleotide as their prosthetic group have already been discussed. The effect of riboflavin deficiency is in all probability not limited to its effect on the *d*-amino acid oxidase alone. Other riboflavin enzyme systems are undoubtedly involved, each deficiency contributing to the sum total of physiological effects observed in a riboflavin avitaminosis.

#### SUMMARY

A riboflavin deficiency in the rat results in a lowering of the *d*-amino acid oxidase content of its liver and kidney. The effect is more pronounced in the liver than in the kidney. A marked decrease in the riboflavin content of the liver is also observed. Restoration of the *d*-amino acid oxidase content to its normal value is accomplished by supplementation with riboflavin. When a growth stimulation results from addition of riboflavin alone to a partially synthetic ration deficient in more than one member

of the vitamin B complex, further supplementation with other members of the vitamin B complex facilitates the complete synthesis of the enzyme.

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# PROGRESS OF HYPERVITAMINOSES D<sub>2</sub> AND D<sub>3</sub> AND RECOVERY IN RATS, AS AFFECTED BY DIETARY CALCIUM AND PHOSPHORUS AND VITAMIN A\*

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This study was undertaken in order to determine whether the findings of Harris and Innes (1), Shelling (2), Brown and Shohl (3), and Bills and Wirick (4) concerning the importance of dietary calcium and phosphorus in hypervitaminosis D could be confirmed when purified diets are employed. Most of these investigators used diets of high cereal content and vitamin D preparations of imperfectly known potency. Also we thought that some light on the mode of action of vitamin D in therapeutic amounts might be obtained from the study of the effects of moderately excessive dosages.

In the present study four vitamin D sources were used, (1) a concentrate made from tuna liver oil, containing probably chiefly the product identified by Brockmann and Busse (5) with irradiated 7-dehydrocholesterol, and (2) an irradiated animal sterol product of invertebrate origin (delsterol),<sup>1</sup> both designated vitamin D<sub>3</sub>, (3) an irradiated ergosterol,<sup>2</sup> and (4) crystalline calciferol,<sup>3</sup> both

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<sup>1</sup> We are indebted for this product to Dr. J. Waddell of E. I. du Pont de Nemours and Company, New Brunswick, New Jersey.

<sup>2</sup> This preparation had a value of 1,000,000 U.S.P. units of vitamin D per gm. and was kindly supplied to us by Dr. C. E. Bills of Mead Johnson and Company.

<sup>3</sup> Kindly supplied to us by the Winthrop Chemical Company, Inc.



designated vitamin D<sub>2</sub>. In addition, parathyroid extract<sup>4</sup> was administered to animals fed the same diets of varying calcium and phosphorus content.

Many of the studies of hypervitaminosis D have involved the use of enormous excesses of the vitamin, sometimes in a single dose, as in the work of Ham and Portuondo (6), sometimes over prolonged periods, as in the work of Shelling and Asher (7). In this series the attempt was made to administer the smallest amount of the preparations which could produce detectable toxic effects in a relatively brief period. Two experiments were performed, hereafter designated Experiments 1 and 2.

In *Experiment 1* eight litters of rats comprising 77 animals were placed when 28 days old, weighing 54 to 66 gm., on the basal diet made up as follows: egg albumin 10, wheat gluten 10, agar 2, Criseo 15, corn-starch 59, and salts 4 per cent.

Three salt mixtures were used, so that Diet 5 contained optimal amounts of calcium and phosphorus, Diet 4 no calcium, optimal phosphorus, and Diet 3, no phosphorus, optimal calcium. The composition of these salt mixtures and other details of procedure have been published previously (8). Harris yeast vitamin extract 100 mg. and crystalline thiamine chloride 5  $\gamma$  per rat per day were given as sources of the B vitamins.

The litters were divided as evenly as possible as to sex and weight into three groups to be fed these diets and each of these groups then subdivided into other groups to receive the vitamin D or parathyroid extract supplements. The supplements, per gm. of body weight per day, were as follows: Group 1,  $\beta$ -carotene in corn oil, 125  $\gamma$  equivalent to 75 units of vitamin A; Group 2, tuna liver oil, 75 units of vitamin A and 63 units of vitamin D<sub>3</sub>; Group 3, irradiated ergosterol, 200 units of vitamin D<sub>2</sub>, and carotene equivalent to 75 units of vitamin A; Group 4, parathyroid extract 0.4 unit, carotene equivalent to 75 units of vitamin A. In addition two groups of controls were fed the low calcium and low phosphorus diets and given (Group 5) carotene in corn oil, 34  $\gamma$  equivalent to 20 units of vitamin A per rat per day; Group 6, cod liver oil, 10 units of vitamin D<sub>3</sub> and 50 units of vitamin A.

In *Experiment 2*, 109 rats from eleven litters were similarly

<sup>4</sup> Kindly supplied by E. R. Squibb and Sons.

divided, placed on the basal diet, and one animal from each litter sacrificed at once in order to determine tissue composition at the outset. The three diets of varying calcium and phosphorus content were again used but this time an attempt was made to equalize the Mg content of all the diets. The vitamin B complex was supplied from thiamine chloride 5  $\gamma$ , rice bran concentrate 100 mg., riboflavin 10  $\gamma$ , and Harris yeast concentrate 100 mg. per rat per day. This change was made because in Experiment 1 certain changes in the fur and skin indicated possible slight deficiencies in some of the vitamin B factors. The supplements of vitamin A were supplied by a refined soup-fin shark liver oil<sup>5</sup> which contained 130,000 U.S.P. units of vitamin A and 85 units or less of vitamin D per gm. The activated animal sterol (delsterol) was used as source of vitamin D<sub>3</sub>, a product which contained 300,000 units of vitamin D per gm. As source of vitamin D<sub>2</sub> pure crystalline calciferol was used. All of these products were assayed in our laboratory by the U.S.P. XI official methods for vitamins A and D.

The supplements were given to the groups in the following amounts: Group 1, standard cod liver oil providing 10 units of vitamin D<sub>3</sub> and 80 units of vitamin A per rat per day; Group 2, 200 units of vitamin A from shark liver oil and its accompanying 0.1 unit of vitamin D<sub>3</sub> per gm. of body weight per day; Group 3, 200 units of the vitamin D<sub>3</sub> (activated animal provitamin D in corn oil) per gm. of body weight per day and 10 units (total per rat) of vitamin A from the shark liver oil; Group 4, 200 units of the same vitamin D<sub>3</sub> and 200 units of vitamin A from refined soup-fin shark liver oil per gm. of body weight per day; Group 5, 200 units of vitamin D<sub>2</sub> as crystalline calciferol and 200 units of vitamin A from shark liver oil per gm. of body weight per day.

The calcium, magnesium, and phosphorus contents of these diets are shown in Table I.

*Procedure in Experiment 1*—After 4 weeks all surviving animals were killed, serum calcium and inorganic phosphorus determined, one tibia of each saved for examination, and the femurs dried, extracted with alcohol and ether, ashed, and P, Ca, and Mg contents determined. The heart, one lung, and one kidney were

<sup>5</sup> The shark liver oil was given us by Mr. T. Sanford of the F. E. Booth Company, Berkeley, California.

dried, ashed, and analyzed for Ca and P content. The method of Fiske and Subbarow (9) was used for P determinations, that of Larson and Greenberg (10) for Ca; the Mg was determined by precipitation as magnesium ammonium phosphate, followed by determination of the P content of the precipitate by the method of Fiske and Subbarow. The other kidney and lung were sectioned and examined microscopically. The food intake was carefully recorded and weight gains or losses considered in relation to it.

The unusually good growth of the group on the low calcium diet, fed high carotene and no vitamin D, led us to question whether the extra vitamin A was of significance. We then placed sixteen more animals on the low calcium and low phosphorus

TABLE I  
*Mineral Content of Diets*

Diet No.	Experiment No.	Ca	Mg	P	Ca:P	Designation
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
5	1	0.51	0.020	0.34	1.5	Normal
	2	0.54	0.033	0.46	1.2	
4	1	0.025	0.127	0.34	0.07	Low Ca-adequate P
	2	0.037	0.037	0.47	0.08	
3	1	0.64	0.046	0.025	25.6	Adequate Ca-low P
	2	0.79	0.038	0.032	24.7	

diets with low carotene (Group 5) and normal vitamin intake from cod liver oil (Group 6).

*Procedure in Experiment 2*—Several of the animals in each group were killed after 28 days and in addition to the same examinations listed for Experiment 1 vitamin A analyses of the liver were also made. Part of those remaining in each group were continued on the same régime for another 28 days, then sacrificed. From the diet of the rest in each group the excess vitamin D was removed from the 28th to the 56th day, so that the rate of recovery from the hypervitaminosis might be noted.

*Growth and Food Utilization*—As may be seen in Fig. 1, the growth of all groups in Experiment 1 on the normal diet except that given excess irradiated ergosterol was satisfactory although not optimum. The gain per 100 gm. of food eaten was nearly the

same, 23 gm. in the high carotene, tuna liver oil, and parathyroid groups. Those given irradiated ergosterol lost weight, although they ate about half as much as the others, and two died after 14 and 21 days.

The rats on the low calcium diet with tuna liver oil and irradiated ergosterol grew nearly as well as those on the normal diet, particularly the former group, and the parathyroid-treated

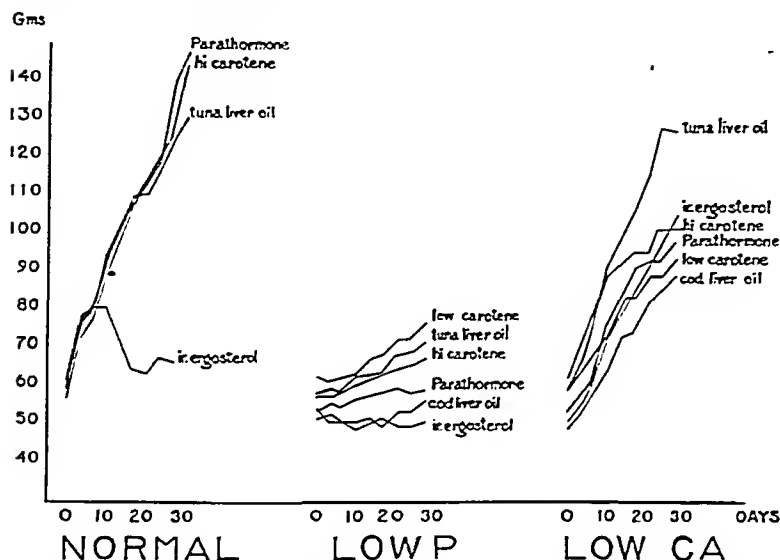


FIG. 1. Growth of rats used in Experiment 1. Normal, Diet 5; low P, Diet 3; low Ca, Diet 4. The groups received the following: (Group 1, *hi carotene*) carotene only; excess tuna liver oil (Group 2); excess irradiated ergosterol and carotene (Group 3); carotene only, parathyroid extract injected (Group 4, *parathormone*); small amount of carotene only (Group 5, *low carotene*); therapeutic amount of cod liver oil (Group 6).

animals also grew, but more slowly. Food utilization was about alike in these three groups, 21 to 24 gm. gain per 100 gm. of intake. The low carotene and cod liver oil groups on the other hand grew only about one-half as much, although food intake was nearly the same. None died during the course of the experiment and all appeared in good condition.

The rats on low P diets on all vitamin doses grew almost not at all and ate much less than the others. The group given ir-

radiated ergosterol was in the worst condition and one died after 11 days.

Clearly either low vitamin D or excessive D<sub>2</sub> or D<sub>3</sub> intake was of no advantage to the rats fed the low phosphorus diet but decidedly favored total growth in the animals on the low calcium diet.

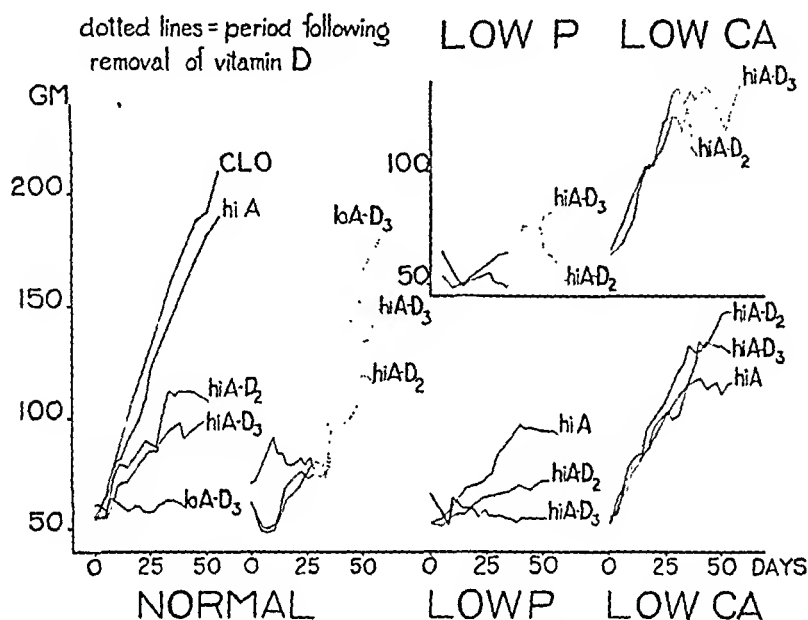


FIG. 2. Growth of rats used in Experiment 2. Normal, Diet 5; low P, Diet 3; low Ca, Diet 4. The groups received the following: cod liver oil in therapeutic amount (Group 1, CLO); shark liver oil only (Group 2, hiA); excess delsterol and a small amount of shark liver oil (Group 3, loA-D<sub>3</sub>); excess delsterol and excess shark liver oil (Group 4, hiA-D<sub>3</sub>); excess calciferol and excess shark liver oil (Group 5, hiA-D<sub>2</sub>).

In Experiment 2 quite similar results were seen (Fig. 2) but the greater excess of vitamin D<sub>3</sub> (delsterol), compared with the tuna liver oil concentrate used in Experiment 1, produced growth and food utilization results on all the diets like those of the excess vitamin D<sub>2</sub> but was usually more unfavorable. A striking difference was seen, however, in the recovery rates after the excess vitamin was discontinued. The groups which had had excess vitamin D<sub>3</sub> recovered in all cases more rapidly and completely than did those which had had the excess D<sub>2</sub>. This was especially

TABLE II

*Serum Composition and Femur Ash of Rats Given Excess Amounts of Vitamin D from Various Sources*

Experiment No.	Diet	Group No. and supplements	No. of rats	Length of feeding period	Serum, mg. per 100 ml.		Femur ash, per cent of dry fat-free bone
					Ca	P	
1	5. Normal	1. Carotene	5	30	9.7	9.0	55.2
		2. Tuna liver oil	5	30	15.0	8.8	52.9
		3. Irradiated ergosterol and carotene	5	20	18.2	7.6	44.7
		4. Parathyroid extract and carotene	5	30	14.9	8.3	53.8
	3. Low P	1. Carotene	5	30	13.8	2.8	41.5
		2. Tuna liver oil	5	28	13.5	3.1	41.6
		3. Irradiated ergosterol and carotene	5	25	14.2	2.9	41.1
		4. Parathyroid extract and carotene	5	29	13.3	3.7	45.8
	4. Low Ca	5. Low carotene	4	30	14.4		42.6
		6. Cod liver oil	4	30	13.8		45.5
		1. Carotene	5	30	7.9	8.3	39.1
		2. Tuna liver oil	5	30	12.3	9.1	36.1
		3. Irradiated ergosterol and carotene	5	29	13.4	9.3	35.4
		4. Parathyroid extract and carotene	6	30	10.0	8.3	36.8
		5. Low carotene	4	30	8.1		37.4
		6. Cod liver oil	4	30	8.5		34.6
2	5. Normal	1. Cod liver oil (control)	3	28	12.0	5.9	55.8
			7	56	9.3	6.8	62.7
		2. Shark liver oil (control)	3	28	10.5	6.7	55.3
			5	56	10.7	8.4	56.4
		3. Excess delsterol and low vitamin A	4	28	14.3	10.6	43.8
		(from shark liver oil)	3	56	13.9	10.7	40.9
			2	56*	10.9	7.8	57.9
		4. Excess delsterol and shark liver oil	4	28	12.3	6.7	47.1
			3	56	15.4	9.6	47.7
			2	56*	13.0	7.7	57.3
		5. Excess calciferol and shark liver oil	4	28	15.2	6.7	46.3
			3	56	15.9	10.9	51.6
			2	56*	10.9	9.7	54.0

TABLE II—*Concluded*

Experiment No.	Diet	Group No. and supplements	No. of rats	Length of feeding period	Serum, mg. per 100 ml.		Femur ash, per cent of dry fat-free bone
					Ca	P	
2	3. Low P	2. Shark liver oil (control)	4	28	9.2	6.2	41.3
			5	56	12.4	6.4	49.8
		4. Excess delsterol and shark liver oil	7	28	13.0	6.2	43.0
			2	56	14.6		48.8
			2	56*	14.1	6.7	41.7
			4	28	10.5	4.0	44.0
		5. Excess calciferol and shark liver oil	3	56		8.8	46.8
			2	56*		8.3	45.5
	4. Low Ca	2. Shark liver oil (control)	4	28	6.3	6.3	37.7
			4	56	9.5	9.3	38.7
		4. Excess delsterol and shark liver oil	3	28	10.2	6.7	34.2
			3	56	13.9	11.6	36.7
			2	56*	10.4	11.5	36.0
			3	28	7.9	6.0	36.7
		5. Excess calciferol and shark liver oil	3	56	10.6	10.7	39.5
			2	56*		6.8	36.1

\* Excess vitamin D discontinued after the 28th day.

notable in the case of the group on the low phosphorus diet. The animals on the low calcium diet on the other hand were all unfavorably affected by the discontinuance of the excess vitamin D in both forms.

*Femur Ash*—As shown in Table II, for Experiment 1 all groups on the normal diet except those given excess vitamin D<sub>2</sub> had normal bone ash content, 52 to 55 per cent. On excess vitamin D<sub>2</sub> this was lowered to 44.7. The bone ash of all those on the low phosphorus diet was low, 41 to 46 per cent. The rats given cod liver oil in small quantity had 45.5 per cent bone ash and those given parathyroid extract 45.8 per cent. These two groups were also in better condition throughout than the others. All rats on the low calcium diet had poorly calcified femurs, 34 to 39 per cent ash, the two carotene groups having the highest.

In Experiment 2 (Table II and Fig. 3) similar conditions were found except that the excess vitamin D<sub>3</sub> (as delsterol) as well as D<sub>2</sub> caused the same decreases in ash content of the femurs of the

animals fed the normal diet. The group with low vitamin A and excess  $D_3$  had lower femur ash than any of the others. When the excess vitamin D was discontinued, an increase in femur ash occurred on the normal diet, greater in the vitamin  $D_3$  than in the  $D_2$  groups. Again the low P diets produced femur ash values of 41 to 49 per cent, with little discernible effect of the excess vitamin D. On the low Ca diets again the femur ash was 34 to 39 per cent and the excess vitamin D had little effect. Apparently the lack of the necessary Ca and P in the diets has so overwhelming an effect on the bone calcification as to mask the excess vitamin D effect if such exists.

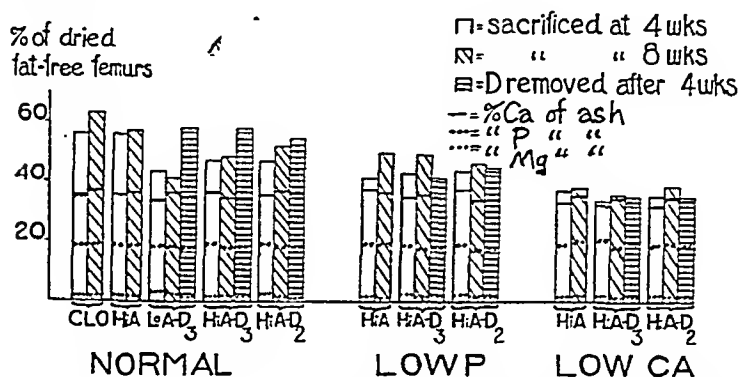


FIG. 3. Femur ash and its content of Ca, Mg, and P (Experiment 2) affected by dietary Ca and P and vitamin D. The groups and diets are designated as in Fig. 2.

In spite of wide variations in the ash content of the femurs of different groups, the Ca, Mg, and P composition of the ash remained remarkably constant (Fig. 3).

*Serum Composition*—Total Ca and inorganic P of the sera were determined in all groups with the results shown in Table II. On normal and low Ca diets the serum P varied very little, being 7 to 9 mg. per cent. But on the low P diet in Experiment 1 very low values were seen, all being close to 3 mg. per cent, with the parathyroid group highest. In Experiment 2 all the values for serum P were fairly close together and higher, up to 8.8 mg. per cent.

In general, serum Ca was most increased by excess vitamin D



and parathyroid extract in Experiment 1 on the normal diet and least on the adequate Ca-low P diet. Slight hypercalcemia existed in rats fed the low P diet even without excessive vitamin D supplements. The excess vitamin D had little effect on the already heightened level. Low levels of serum Ca occurred on the low Ca diet in all but in the excess vitamin D<sub>2</sub> and D<sub>3</sub> groups and in these only slight hypercalcemia occurred. In Experiment 2 the same range was found.

The relatively mild effect of parathyroid extract and vitamin D on serum Ca in the group on the low P diet confirms the findings of earlier experiments in this laboratory (8, 11) on rats and dogs in which rachitogenic cereal and purified diets were used. Combination of the two substances, vitamin D and parathyroid extract, was found in these former studies to produce immediate and large increases in serum Ca.

*Liver Vitamin A*—The reserves of vitamin A in the livers of all animals used in Experiment 2 were measured by the antimony trichloride test in which the Lovibond tintometer and the procedure of Davies (12) were employed. Expressed as blue units per gm. of fresh liver, the amount of vitamin A found in all groups was proportional to the intake. All animals which received the large daily ration of 200 units of vitamin A per gm. of body weight had 4000 to 22,000 blue units per gm. of liver, with most of the values in the upper range. The concurrent excess of vitamin D<sub>2</sub> or D<sub>3</sub> appeared to exert little influence on the vitamin A storage. In the two groups which received lower amounts of vitamin A, a total of 70 units from cod liver oil and 10 units from shark liver oil per rat per day, both with normal diet, the liver vitamin A was strikingly lower, 1400 blue units per gm. of liver at 28 days and 480 units at 56 days in the former and 11 to 59 units in the latter.

*Tissue Calcium and Phosphorus*—None of the soft tissues of animals of the low Ca group in Experiment 1 showed abnormal values of Ca and P (Fig. 4) nor did those from animals on the normal diet except in the groups given excess vitamin D<sub>2</sub>. However, some increase in tissue Ca, particularly in the kidneys, was seen in all but the high carotene group of the rats fed the low P diet. This was especially striking in the low carotene group, a finding which confirms the impression that kidney stone forma-

tion may result from even border line deficiency of vitamin A. The most potent combination for the formation of kidney calcification would appear to be the low P, excess vitamin D, low vitamin A diet. The parathyroid treatment was no more effective in producing tissue calcification than the relatively mild hypervitaminosis produced by the tuna liver oil. Actual stones were found in the kidneys of some of these rats fed low carotene and excess vitamin D<sub>2</sub> but diffuse calcareous deposits were more generally seen.

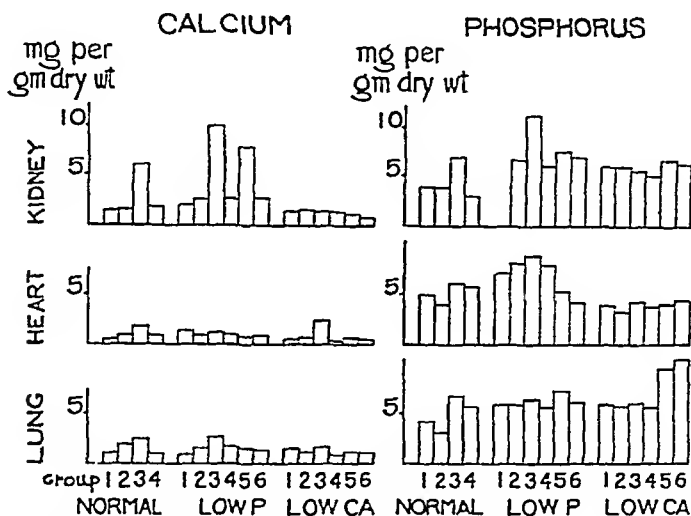


FIG. 4. Ca and P of hearts, lungs, and kidneys of rats used in Experiment 1. The groups and diets are designated as in Fig. 1.

In Experiment 2 these findings were confirmed. The striking rise in all soft tissue calcium (Fig. 5) of the rats on the normal diet with low vitamin A and excess delsterol overshadowed the rises in all other groups. The delsterol excess on all diets produced more soft tissue calcification than did the vitamin D<sub>2</sub> and this difference was intensified when the experimental period was prolonged to 56 days. But when the excess vitamin D was discontinued after 28 days, resolution of tissue calcification was prompt and complete in the normal and low P groups which had been given delsterol but slow and incomplete in those which had received excess vitamin D<sub>2</sub>. In all cases the rats fed the low Ca

diet and excess vitamin D<sub>2</sub> or cholesterol had no abnormal tissue calcification except *after withdrawal* of such excess.

The low P diet produced higher tissue P (Fig. 6) than did normal or low Ca diets except in the lungs of the low vitamin A and cod liver oil groups on the low Ca diet in Experiment 1, and in the lungs and kidneys of the low vitamin A group on the normal diet in Experiment 2. The P content of the soft tissues varied much less than did the Ca in both experiments and on all diets. If all mineral deposits are reckoned as Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, the percentage in-

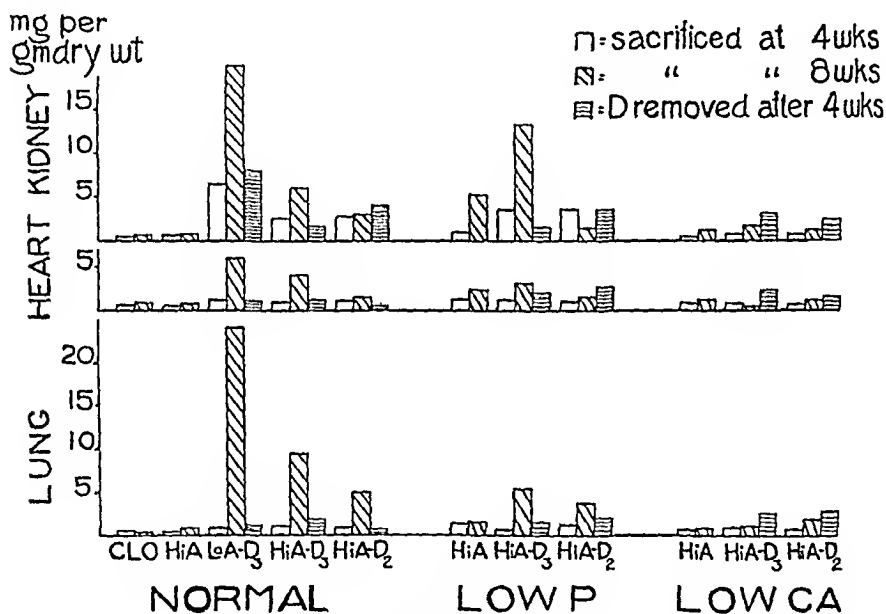
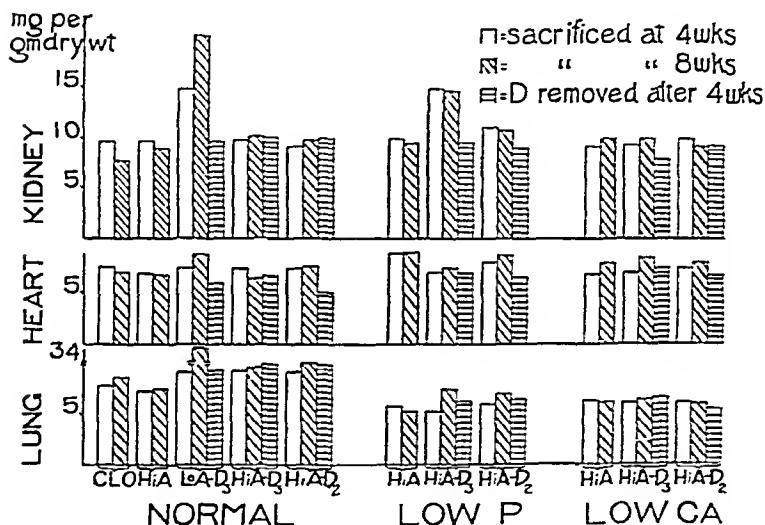


FIG. 5. Ca of hearts, lungs, and kidneys of rats used in Experiment 2. The groups and diets are designated as in Fig. 2.

crease in P required as an accompaniment to even a large relative increase in Ca is negligible. For instance the increase of Ca in kidneys and lungs of the low vitamin A-high vitamin D<sub>3</sub> group from 0.5 mg. per gm. of dry tissue to 22 and 24 mg. (a 44- or 48-fold increase) produced an increase in P to 19 and 35 from the normal of 9 mg. per gm. of dry tissue, only a 2- or 4-fold increase. The calculated increase in P if the added Ca was laid down as Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> is 11 or 12 mg., making the total 21 or 22 mg., to be compared with 19 and 35 mg. found.

*Microscopic Appearance of Tissues*—Histological study of kid-

neys and lungs yielded the interesting observation that many kidney tubules of rats without vitamin D on the low Ca diet were extensively dilated, with reduction of the epithelium to a thin lining. Other tubules showed obliteration of lumen and were undergoing degeneration. No calcareous deposits were seen. On the same diet with excess vitamin D the tubules were more nearly normal. Most of the low Ca group had enlarged fatty livers.



Ca group but calcified spots on the kidney in the other two diet groups.

*Kidney Enlargement*—Hypertrophy of the kidneys occurred in all of the animals which were unfavorably affected by the hypervitaminosis. The ratio of the weight of the fresh kidneys to the body weight of normal rats is stated by Donaldson (13) to be 4.2 to 5.5 mg. per gm. of body weight. The rats on the normal diet without excess vitamin D in Experiment 2 had a kidney-body weight ratio of 4.4 to 5.3. With low vitamin A and excess vitamin D<sub>3</sub> the ratio was 10.0 and on discontinuance of the excess D<sub>3</sub> it was lowered after 28 days to 4.6. The groups with excess vitamin A as well as excess D had kidney ratios of 6.0 to 7.6. On the discontinuance of the excess vitamin D<sub>3</sub> the ratio fell to 4.8 but discontinuance of the excess D<sub>2</sub> had no effect. On the low P diet the kidney ratios with and without excess vitamin D ranged from 6.9 to 11.8 and on the low Ca diet from 5.4 to 6.5. In most cases the ratio was in the normal range on the low Ca diet but was in all cases indicative of hypertrophy on the low P diet. In the low Ca group on the other hand the tubules were enormously dilated, with typical hydronephrosis, the kidney proper appearing to be a mere shell, except when excess vitamin D was given. As shown by Day and McCollum (14), on low P intake large amounts of Ca are excreted by the kidney but on low Ca intake P is excreted in large amount in the urine (15). Apparently the excess Ca excretion promotes renal hypertrophy more than does P excretion but the latter is more damaging to the kidney tissue. MacKay and Oliver (16) reported kidney hypertrophy and highly specific tubule damage in rats when the inorganic phosphate of the diet was considerably raised. There was no decrease in the calcium content of their diets, however, as there was in the experiments here reported. The same kind and location of damage to the tubules were found in our rats fed low Ca. Connective or fibrous areas were seen in the kidneys of rats which had been allowed to recover from the hypervitaminosis D.

#### DISCUSSION

These findings do not support the conclusion that vitamin D operates solely by increasing the net absorption of Ca or P or of

both from the intestine nor do they indicate that serious derangement of the Ca : P ratio may be counterbalanced or even much affected by vitamin D intake. On both low P and low Ca diets there was no obvious improvement in bone calcification by either excessive or moderate doses of vitamins D<sub>2</sub> or D<sub>3</sub>. The improvement in growth of soft tissue seen in the high vitamin D groups on the low Ca diet compared with the group on the same diet without vitamin D must be the result of better P utilization, although dietary P and serum P were both adequate in the control animals which showed no such improvement. Obviously no improvement in bone structure or Ca use occurred, although serum Ca was raised to normal in the better growing animals on excess vitamin D.

There was no improvement in growth, bone ash, or serum P in the rats fed the low P diet with the excess vitamin D and only slight improvement in those given either parathyroid extract or small amounts of cod liver oil. This is in striking contrast with the effects many times seen in animals on cereal diets of low P. The explanation offered by Schneider and Steenbock (17) for this difference which they observed also with similar diets but without use of excess vitamin D is that the total quantity of P retained on the cereal diet (Ration 2965) is large enough to provide for both bone and soft tissue P but on their extremely low P purified diet the absolute quantity of P retained was too small to allow soft tissue growth and instead was used only for bone formation. Day and McCollum (14), on the other hand, with similar but more severely P-low diets found only negative P balances, even though adequate amounts of vitamin D were given and there was some growth for 5 or 6 weeks. They conclude that the P for soft tissue growth was supplied by the bone.

As may be seen in Figs. 4 and 6, there was no consistent change in the P content of any of the tissues of our rats and there was no marked improvement in either bone formation or soft tissue growth due to either normal or moderately excessive vitamin D intake on the low P and normal diets. On the other hand with the low Ca diet the excess vitamin D promoted soft tissue growth but was without benefit to bone formation.

There would appear then to be only one diet, the rachitogenic cereal diet, with which it has been shown that vitamin D effects

significant increase in bone calcification in rats. Schneider and Steenbock (17) obtained 46 per cent increase in P content of bone, from 44.1 to 64.5 mg. per gm. of dry weight, when 50 units of vitamin D<sub>2</sub> per day were fed for 2 weeks to rats on the cereal diet but only 20 per cent, *i.e.* from 36.9 to 44.5 mg. per gm. of dry weight, under the same conditions in rats fed their purified low P ration. The failure of growth which they observed in the latter group was also manifest in all our rats given excess vitamin D except those on the low Ca diet. But withdrawal of P from soft tissues for bone growth could not, as they suggest, explain this stoppage of growth in our groups which received the normal diet. Withdrawal of Ca from bone, however, might be assumed to occur in the low Ca-fed rats which grew under the influence of vitamin D if Ca were required in any significant amounts for soft tissue growth. Certainly the bone ash of these rats was decreased, while the soft tissue weight was increased. The condition of the kidneys also indicated that conservation of dietary P for soft tissue growth was promoted by vitamin D on the low Ca diet.

If vitamin D increases Ca absorption, it should improve bone formation on low Ca diets and should have little effect on soft tissue growth, the latter requiring chiefly P mobilization. But the conditions observed were the opposite, failure of bone calcification and improved soft tissue growth.

If vitamin D mobilizes P from soft tissues, it should improve bone formation and retard soft tissue growth on the low P diet. But only depression of growth was noted.

If parathyroid extract has no effect on Ca absorption but merely increases P excretion as is often stated (Albright, Sulkowitch, and Bloomberg (18)), the improved bone ash of the parathyroid group on the low P diet and the general resemblance of the findings on these animals to those of the cod liver oil and tuna liver oil groups are hard to understand. Parathyroid extract should exaggerate the bad effects of a low P diet and mitigate those of a high P (relative to Ca) diet. But the opposite conditions appeared in this study.

On each of the three diets the groups treated with parathyroid extract and those given a moderate excess of vitamin D<sub>3</sub> in tuna liver oil and a minimum amount in cod liver oil presented nearly

the same results. This was true of the rats given the low Ca diet, even though the serum Ca produced in these three groups varied, 10.0, 12.3, and 8.5 respectively.

*Recovery from Hypervitaminosis*—On the two diets, normal and low P, with which excesses of vitamin D produced obvious symptoms of hypervitaminosis in Experiment 2, vitamin D as delsterol caused greater damage than did corresponding excess of vitamin D<sub>2</sub> (as irradiated ergosterol or calciferol). More animals died, less growth was obtained, and there were more soft tissue calcification and less femur ash. During the recovery period, however, quite opposite results were seen. The rats relieved of excess delsterol began to grow rapidly and after 28 days presented better conditions in both soft tissues and bones than the corresponding groups relieved of excess vitamin D<sub>2</sub>. This was particularly obvious in the rats on the diet of low P content. There was no group fed low vitamin A and excess D<sub>2</sub>, but the extremely depressed condition of the group given little vitamin A and excess delsterol was noticeably contrasted with the rapid recovery of all these animals when relieved of the latter excess. The groups which received the excess delsterol and excess vitamin A were much less adversely affected and also made rapid recovery when the excess D was removed from the diet. Those which received vitamin D<sub>2</sub> (calciferol) had somewhat less rapid and profound failure of appetite and growth and less tissue damage than did those receiving delsterol but those which were relieved of the excess recovered more slowly. In Fig. 5 the amount of soft tissue calcification is seen to illustrate this difference. The corresponding lowering of the femur ash, in inverse order, is shown in Fig. 3. Tissue P varied less consistently with the progress of the hypervitaminosis. The soft tissue P tended to be constant, slightly higher on the low P diet than on the other diets, and not increased significantly by excess vitamin D except in the group with low vitamin A intake.

The entire picture of progress of, and recovery from, the hypervitaminosis is consistent with the view that the irradiated animal sterol (delsterol, vitamin D<sub>3</sub> ?) is more rapidly and completely absorbed than is irradiated ergosterol (calciferol, vitamin D<sub>2</sub>) and is likewise more rapidly and completely excreted. Studies of fecal and blood vitamin D during and after administration of



moderately excessive doses of these two substances are now under way in an effort to confirm this hypothesis.

Earlier experiments (19) in this laboratory conducted in a similar way but with only the normal diet, irradiated ergosterol, and fish liver oil concentrates yielded results which justified the conclusion that the hypervitaminosis produced by the former was the more severe, although the difference was not striking when the accompanying vitamin A intake was equalized. The greater severity of the symptoms produced by delsterol in the experiments here reported when compared either with the effects of excess tuna liver oil in this and in the previous studies or of calciferol and irradiated ergosterol in the present study is difficult to explain unless delsterol is not identical with the provitamin D (D<sub>3</sub>) of tuna and cod liver oils.

#### SUMMARY

1. The levels of both calcium and phosphorus and their ratio in the diet were the primary factors in production of normal femur calcification of young rats whether normal or excess amounts of vitamins D<sub>2</sub> and D<sub>3</sub> were given or chronic moderate hyperparathyroidism was produced by injection of parathyroid extract.

2. The damaging effects of the low phosphorus, purified diet were not mitigated by administration of either excess or minimum doses of vitamin D or parathyroid extract. This is in contrast to the results seen with high calcium-low phosphorus cereal diets.

3. The low calcium diet did not produce normal bone under any of these conditions but with excess vitamin D produced more nearly normal soft tissue growth. In addition, normal kidney structure occurred in the latter groups but damaged tubules in the controls without vitamin D.

4. Chronic mild parathyroid treatment (0.4 unit per gm. of body weight daily) without vitamin D had but little harmful effect with any of these diets and seemed to be advantageous with the low phosphorus diet, exerting a similar effect to that of normal amounts of cod liver oil.

5. Excessive amounts (200 U.S.P. units per gm. of body weight daily) of irradiated ergosterol or calciferol were most harmful with a diet of normal calcium and phosphorus content and ratio, and had toxic effects also with the adequate calcium-low phos-

phorus diet but only favorable results with the low calcium-adequate phosphorus diet. The same amounts of irradiated animal sterol (delsterol, presumably vitamin D<sub>3</sub>) produced similar but even more striking damage.

6. Recovery from the toxic effects of excess of vitamin D as delsterol was more rapid and complete than from those produced by excess vitamin D<sub>2</sub>. This is interpreted to mean that in the rat both absorption and excretion of the vitamin D<sub>3</sub> are more efficient than is the case with vitamin D<sub>2</sub>.

7. Low vitamin A intake allowed greatly increased tissue calcification and other damaging effects of hypervitaminosis D.

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### DIMETHYL SULFONE; A CONSTITUENT OF THE ADRENAL GLAND

Sirs:

Ruzicka, Goldberg, and Meister<sup>1</sup> have just described the isolation of dimethyl sulfone from the dried blood of cattle. We have encountered this compound several times in the preparation of adrenal steroids from fresh beef glands.<sup>2</sup> In the purification of the cortical hormones by the ether-acid-ether procedure<sup>3</sup> the sulfone is concentrated in the aqueous acid. On washing with chloroform, a highly reducing fraction is obtained which yields the crystalline sulfone from a concentrated alcoholic solution. 186 mg. of the crude crystalline sulfone were obtained from 1000 kilos of glands. This represents about one-half the yield obtained by Ruzicka *et al.* from blood. The actual concentration of the compound in blood and tissues is no doubt much greater.

Our preparation was purified by repeated sublimation in a vacuum and crystallization from ethyl alcohol. It then melted at 108°. In admixture with a synthetic sample of dimethyl sulfone (m.p. 109°) it melted at 109°. Analysis, calculated for  $C_2H_6O_2S$ , C 25.51, H 6.43, S 34.07; found, C 25.71, 25.70, H 6.03, 6.23, S 33.68, 33.89.

The occurrence of dimethyl sulfone in the adrenal gland is of particular interest, since Reichstein<sup>4</sup> has reported the isolation of bis- $\beta$ -hydroxy ethyl sulfoxide from extracts of this organ. We have also isolated taurine and ethyl hydrogen sulfate as its potas-

<sup>1</sup> Ruzicka, L., Goldberg, M. W., and Meister, H., *Helv. chim. acta*, **23**, 559 (1940).

<sup>2</sup> Paper read before the Michigan Academy of Science, Arts and Letters, March 17, 1939.

<sup>3</sup> Pfiffner, J. J., and Vars, H. M., *J. Biol. Chem.*, **106**, 645 (1934). Pfiffner, J. J., Wintersteiner, O., and Vars, H. M., *J. Biol. Chem.*, **111**, 585 (1935).

<sup>4</sup> Reichstein, T., *Helv. chim. acta*, **19**, 41 (1936). Reichstein, T., and Goldschmidt, A., *Helv. chim. acta*, **19**, 401 (1936).

sium salt. Still another sulfur compound has been obtained in crystalline but as yet impure form. It is seemingly new to the animal body. We hope to report on its identity in the near future.

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## THE ABSORPTION OF WATER-SOLUBLE VITAMIN K WITHOUT THE AID OF BILE SALTS\*

Sirs:

It has been shown experimentally that the fat-soluble forms of vitamin K are not readily absorbed from the intestine unless bile salt is present.<sup>1</sup> However, there is no evidence as to whether bile salts are of importance in the utilization of the water-soluble forms of this vitamin. We wish, therefore, to present certain data bearing on this point.

The compound used by us was the water-soluble hydrochloride of 4-amino-2-methyl-1-naphthol. It has been shown<sup>2</sup> that the activity of this compound is but little greater than that of the fat-soluble forms of the vitamin. A daily dose of 1 mg. was therefore deemed suitable, and this amount was given daily to a number of patients suffering from chronic obstructive jaundice. In one case the plasma prothrombin was 14 per cent of normal prior to administration of the compound. On the following day, it rose to 63 per cent of normal, on the 3rd day to 82 per cent, and 4 days later to 94 per cent of normal. In the second case, the prothrombin rose from 54 per cent to a level of 80 per cent in 2 days, and 5 days later a level of 107 per cent was recorded. In the third case, the initial prothrombin level was 37 per cent of normal. After 1 day of treatment, it rose to the 88 per cent level; on the 2nd day, the reading was 81 per cent, and on the 4th day 103 per cent.

The rapid response in the plasma prothrombin level in these

\* Aided by a grant from the John and Mary R. Markle Foundation, and by funds supplied by the Graduate College, State University of Iowa. The 4-amino-2-methyl-1-naphthol used in these studies was supplied through the courtesy of Parke, Davis and Company.

<sup>1</sup> Greaves, J. D., and Schmidt, C. L. A., *Proc. Soc. Exp. Biol. and Med.*, 37, 43 (1937). Smith, H. P., Warner, E. D., Brinkhous, K. M., and Seegers, W. H., *J. Exp. Med.*, 67, 911 (1938).

<sup>2</sup> Emmett, A. D., Kamm, O., and Sharp, E. A., *J. Biol. Chem.*, 133, 285 (1940).

eases shows that the compound is readily absorbed and that bile salt medication, which tends to create nausea in the patient, is not necessary. These findings supplement the existing theory regarding vitamin K absorption. It is evident that the average diet does not contain any great amount of such water-soluble compounds; otherwise, patients with obstructive jaundice would not develop signs of vitamin K deficiency.

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## THE BIOLOGICAL ORIGIN OF THE AMIDINE GROUP IN CREATINE

Sirs:

Glycine has been shown, with the aid of isotopes, to be the source of the glycine moiety of the creatine molecule, while the nitrogen of the amidine group of the latter compound is derived from the  $\alpha$ -nitrogen of other amino acids.<sup>1</sup> It has now been found that the amidine group of arginine serves as an intermediate in the biological synthesis of creatine.

l(+)-Arginine with N<sup>15</sup> in the amidine group was prepared by degradation of  $\alpha$ -toluenesulfoarginine with baryta to  $\alpha$ -toluenesulfoornithine, treatment of the latter with isotopic methyl isourea (prepared from isotopic cyanamide), and hydrolysis of the resulting toluenesulfoarginine. The arginine had a rotation in 4 N HCl of  $[\alpha]_D^{22} = +25.0^\circ$ , and an isotope content of 8.33 atom per cent N<sup>15</sup> excess.

The addition of a small amount of this material to the stock diet of normal full grown rats resulted in the appearance of muscle creatine with an isotope content far higher than that found after administration of isotopic ammonia, urica, or of any other investigated amino acids, except glycine. It was so high that the amidine group of creatine must have originated from that of arginine, which has recently been shown<sup>2</sup> to be continuously formed in normal animals from the  $\alpha$ -nitrogen of various amino acids. Both automatic processes, the formation of the amidine group and the amidine transfer from arginine to glycine for creatine synthesis, are thus interlinked.

Bergmann and Zervas<sup>3</sup> 12 years ago suggested the occurrence of this biological transfer of the amidine group on the basis of chemical experimentation. Triacetylanhydroarginine easily

<sup>1</sup> Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 633 (1940).

<sup>2</sup> Schoenheimer, R., and Rittenberg, D., *Physiol. Rev.*, **20**, 218 (1940).

<sup>3</sup> Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **172**, 277 (1927).



yields this group to glycine to form guanidoacetic acid, which has been shown to be one of the intermediates in the biological creatine formation.

Recently Borsook and Dubnoff<sup>4</sup> have put forward evidence that the methyl group of creatine is derived from that of methionine.<sup>5</sup> This finding in conjunction with those obtained with isotopes establishes the biological origin of all parts of the creatine molecule. The carbon chain and 1 nitrogen atom are derived from glycine, the amidine group is derived from arginine, and the methyl group from methionine.

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<sup>4</sup> Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 559 (1940).

<sup>5</sup> The relation of methionine to biological methylation was brought out by the work of du Vigneaud *et al.* (du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939)), who have now extended the work to creatine. By marking the methyl group of this amino acid with deuterium they obtained unequivocal proof of the shift of this group to creatine.

## THE TRANSFER OF THE METHYL GROUP FROM METHIONINE TO CHOLINE AND CREATINE

Sirs:

We have recently shown that homocystine (homocysteine) can replace methionine in the diet of the white rat only in the presence of choline or certain related substances,<sup>1, 2</sup> and have suggested that choline enables the animal to synthesize methionine under these conditions. It was postulated that the fundamental process involved is a transfer of a methyl group from the nitrogen of choline to the sulfur of homocysteine. Furthermore it was suggested that the reaction might be reversible and thus methionine might act as a donor of methyl groups in the synthesis of choline in the body.

It was realized that direct proof of the transfer of the methyl group (transmethylation) was highly desirable. We decided to attempt first to trace the migration of the methyl group from methionine to choline. A methionine was therefore synthesized containing deuterium in the methyl group. The trideuteromethyl iodide used in the synthesis<sup>3</sup> was prepared from deuteromethyl alcohol, which had been made by reduction of CO with D<sub>2</sub> by the method of Zanetti.<sup>4</sup> The trideuteromethionine (23.9 atom per cent D) was fed at an average daily rate of 70 mg. (2.4 mg. of deuterium) for 3 weeks to rats kept on a methionine-choline-free diet. The choline chloroplatinates isolated from the tissues contained 32.2 and 31.7 atom per cent deuterium respectively, repre-

<sup>1</sup> du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **128**, p. cviii (1939).

<sup>2</sup> du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).

<sup>3</sup> du Vigneaud, V., Dyer, H. M., and Harmon, J., *J. Biol. Chem.*, **101**, 719 (1933).

<sup>4</sup> The authors wish to thank Professor Zanetti of Columbia University for allowing them the use of his laboratory and equipment to prepare the deuteromethyl alcohol.

senting 57.2 and 56.3 per cent of the theoretically possible amount if all the methyl groups of the choline had come from the deuteromethionine.

In the original presentation of our data concerning the relationship of methionine to choline and to methylation,<sup>1</sup> it was suggested that "The methionine-homocystine-choline relationship may be significant, not only to sulfur and fat metabolism, but also to other problems of methylation in the animal organism." As a direct extension of this concept, we sought for the presence of the deuteromethyl group in the creatine molecule. The creatine was isolated as creatinine from the muscle tissue of a rat that had been fed the deuteromethionine for 3 days. The deuterium content of the zine chloride complex was found to be 3.4 atom per cent D, which is equivalent to 9.1 per cent of the theoretical amount. A longer term experiment (8 weeks) gave 25.9 atom per cent D, equivalent to 69 per cent of the theoretical amount.

From Borsook's work it might appear that choline unlike methionine cannot yield a methyl group directly to guanidoacetic acid to form creatine.<sup>5</sup> However, if homocysteine could act as an intermediary agent, transmethylation from choline to guanidoacetic acid might take place.<sup>6</sup>

The experiments reported herein provide, in our opinion, unequivocal evidence that methionine may furnish the methyl groups of choline and creatine. The question of the relationship of methionine to other methylations in the body still remains. We would suggest that the presence of methyl groups in a utilizable form, such as in methionine and choline, may be essential in the diet.

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<sup>5</sup> Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 559 (1940).

<sup>6</sup> Of interest in this connection are experiments, as yet unpublished, in which we have found that the administration of choline or methionine brings about resumption of growth after an inhibition of growth caused by feeding guanidoacetic acid to rats on a diet suboptimal in methionine and free of choline.

## PREVENTION OF PEROSIS BY CHOLINE

*Sirs:*

A diet was described<sup>1</sup> which produced a high incidence of perosis in young turkeys. The addition of manganese up to a level of 0.4 per cent  $\text{MnSO}_4$  in the diet was found to have no preventive effect, but yeast gave partial protection. Recently Hogan, Richardson, and Patrick,<sup>2</sup> working with chicks, have also described a situation in which perosis occurring in the presence of manganese was prevented by an organic factor.

In the present investigation a modification of the previous diet<sup>1</sup> was used, consisting of yellow corn-meal 65 parts, dried skim milk 15, washed casein 13, alfalfa meal 1,  $\text{CaCO}_3$  3,  $\text{KH}_2\text{PO}_4$  1,  $\text{NaCl}$  (iodized) 0.5,  $\text{MnSO}_4$  0.1, fish oil blend (3000-A, 400-D) 1 (Diet 161). Baby turkeys were placed on the diet at hatching and were observed at frequent intervals for perosis consisting of a distortion of one or both hock joints in lateral, medial, or anterior directions. Shortening and thickening of the bones of the leg, especially the tarsometatarsus, were accompanying symptoms. Perosis was first noted after 8 to 12 days in birds on the basal diet, and the number of cases increased to reach a maximum at 20 to 30 days, by which time in some experiments all the birds were affected. On partially protective diets, symptoms appeared at about the same time as in the case of the basal diet, and reached a maximum at about 20 days, after which the milder cases frequently retrograded and the hock joints became straight during the next 10 days. A test period of 28 to 35 days was used with ten to eleven birds in each group.

It was found that the addition of a mixture of synthetic thi-

<sup>1</sup> Jukes, T. H., *Poultry Sc.*, 18, 405 (1939).

<sup>2</sup> Hogan, A. G., Richardson, L. R., and Patrick, H., *Proc. Am. Inst. Nutrition, J. Nutrition*, 19, 14 proc. (1940).

amine<sup>3</sup> 1.2 mg., riboflavin<sup>3</sup> 1.8 mg., nicotinic acid<sup>3</sup> 3 mg., pyridoxine (vitamin B<sub>6</sub>)<sup>3</sup> 0.9 mg., pantothenic acid 10 mg., and choline chloride 0.15 gm. to 100 gm. of basal diet gave complete protection. Four of the components of this mixture were then fed separately with the results shown in the accompanying table.

In another experiment a simplified diet was fed consisting of glucose (cerclose) 50 parts, washed casein 30, dried yeast<sup>4</sup> 10, salt mixture<sup>5</sup> 5, soy bean oil 4, fish oil blend 1. Perosis appeared in 9 days, and at 19 days the incidence of perosis was 67 per cent, and the average weight was 131 gm. A second group of turkeys re-

Supplement to 100 gm. Diet 101	Per cent of birds showing perosis at			
	12 days	14 days	19 days	28 days
None.....	71	71	85	85
Synthetic vitamin mixture (see text)...	0	0	0	0
1 mg. thiamine.....	40	70	70	80
10 " nicotinic acid.....	29	57	71	57
10 " pantothenic acid.....	25	50	50	38
0.3 gm. choline chloride.....	0	0	0	0

ceived the same diet plus 0.3 per cent of choline, and at 19 days there was no perosis, and the average weight was 200 gm.

The results indicate that choline was effective in preventing perosis in turkeys under the conditions encountered. Choline promoted growth on a simplified diet.

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<sup>3</sup> Supplied by Merck and Company, Inc., through the courtesy of Dr. J. C. Keresztesy.

<sup>4</sup> Anheuser-Busch Strain G, kindly furnished by Mr. G. F. Siemers.

<sup>5</sup> Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, **42**, 180 (1939).

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